The Interaction of Adherent Invasive *Escherichia coli* with Intestinal Epithelial Cells, *In Vitro* Derived M-Cells and Macrophages

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

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DECLARATION

I hereby declare that this thesis is a presentation of my original work. Wherever contributions of others are involved, every effort has been made to indicate this clearly, with due reference to the literature.

The work was done under the joint guidance of Dr Barry J Campbell, and Professor Jonathan M Rhodes, both of the School of Clinical Sciences, at the University of Liverpool.

For Nellie

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ABSTRACT

The initial lesions observed in Crohn's disease typically occur over lymphoid aggregates in the large bowel and Peyer's patches in the small bowel, where M-cells are present. An increased presence of Adherent and Invasive *Escherichia coli* (AIEC) has been reported in Crohn's disease mucosa. Crohn's disease is common in westernised countries where low levels of soluble fibre are eaten and where the use of emulsifiers in processed food is common. It is possible that dietary factors may be implicated in the pathogenesis of the disease. The aims of this work were to establish if AIEC interact with intestinal M-cells, and to establish the effect that dietary factors have on these interactions.

Crohn's disease AIEC are translocated across M-cells significantly more than control *E. coli* K12, and translocation of Crohn's disease AIEC is significantly higher across M-cells than across control Caco2-cl1 monolayers. Soluble plantain fibre markedly reduced translocation of Crohn's disease AIEC across M-cells, as did soluble broccoli fibre. The food emulsifiers polysorbate-60 and polysorbate-80 led to increased translocation of Crohn's disease AIEC across Caco2-cl1 monolayers, but the effect was less clear across M-cell monolayers.

Crohn's disease AIEC demonstrated the ability to survive and replicate within macrophages at levels significantly greater than control *E. coli* K12. Combinations of the antibiotics ciprofloxacin, tetracycline, clarithromycin, trimethoprim and rifampicin were able to kill Crohn's disease AIEC within macrophages.

This work supports the hypothesis that M-cells play an important role the pathogenesis of Crohn's disease. The replication of AIEC within macrophages may result in the typical granulomatous lesions observed in Crohn's disease. The effect of soluble plant fibres on inhibition of bacterial translocation across M-cells represents a novel effect that might be an important mechanism by which dietary fibre could promote intestinal health.

PUBLICATIONS ARISING FROM THIS THESIS

Papers

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Subramanian S, Rhodes JM, Hart CA, Tam B, **Roberts CL**, Smith SL, Corkill JE, Winstanley C, Virji M, Campbell BJ. Characterization of epithelial IL-8 response to inflammatory bowel disease mucosal *E. coli* and its inhibition by mesalamine. Inflamm. Bowel Dis. 2008 Feb;14(2):162-75.

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Abstracts

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ABREVIATIONS USED IN THIS THESIS

AAL	*Aleuria aurantia* lectin
AIEC	Adherent and Invasive *Escherichia coli*
ANOVA	Analysis of variance
CD	Crohn's disease
CFU	Colony forming units
C_{max}	Maximal (peak) serum concentrations
DMEM	Dulbecco's Modified Eagle Medium
EM	Electron microscopy
FAE	Follicle-associated epithelium
FCS	Foetal calf serum
FISH	Fluorescent *in situ* hybridization
IL	Interleukin
LB	Luria Bertani
M-cell	Microfold cell
MAP	*Mycobacterium avium* subsp *paratuberculosis*
MOI	Multiplicity of infection
NSP	Non-starch polysaccharides
OD	Optical density
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
RPMI	Roswell Park Memorial Institute
Spp.	Species
TEM	Transmission electron microscopy
TEER	Transepithelial electrical resistance

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Chapter 1

Introduction, Hypothesis and Aims

1.1 CROHN'S DISEASE

Crohn's disease (CD) is a chronic inflammatory disorder affecting the gastrointestinal (GI) tract. Typically, any part of the GI tract can be involved, although most commonly the distal ileum and caecum are affected. The disease is characterised by uncontrolled inflammation of the intestinal mucosa, and deep inflammation with granulomata (in about 70% of cases). The mucosal immune system of patients becomes chronically activated, and the intestine remains chronically inflamed.

The symptoms of Crohn's disease vary amongst individual patients; the main gastrointestinal symptoms are cramp-like abdominal pain, diarrhoea, vomiting or weight loss.

The disease was independently described by Thomas Kennedy Dalziel in 1913 [Dalziel 1913], and in 1932 by the American gastroenterologist Burrill Bernard Crohn, after whom the disease was named. Crohn, along with two colleagues, described a series of patients with inflammation of the terminal ileum, the area most commonly affected by the illness [Crohn *et al.* 2000].

Crohn's disease is currently of unknown aetiology. It is considered to be a multi-factorial disease, consisting of interplay between host genetics, aberrant immune responses to environmental factors and to the gut microbiota, and defects in the epithelial barrier.

1.2 EPIDEMIOLOGY

1.2.1 Incidence and prevalence

Studies conducted across the UK (Aberdeen [Kyle 1992] and Cardiff [Thomas *et al.* 1995]), and some parts of Europe (Stockholm [Lapidus *et al.* 1997] and Orebro [Lindberg *et al.* 1991] in Sweden) suggest that the incidence of Crohn's disease rose during the 1950s and 1960s. More recently, some reports suggest that Crohn's disease incidences have been decreasing from north to south across Europe [Stewenius *et al.* 1995; Lapidus *et al.* 1997], whilst other studies have found comparable rates between Southern and Northern Europe [Shivananda *et al.* 1996]. There is considerable difficulty in establishing the true incidence of Crohn's disease across different European countries, due in part to historical and local differences in Crohn's disease diagnosis, and the difficulties in obtaining comparable retrospective data for analyses [Logan 1998].

Within the UK, epidemiological data derived from hospital led studies find the incidence of Crohn's disease to be 6.7 per 100,000 annually (range 1.6 to 14.6), with a prevalence of 140 cases per 100,000 people (range 10 to 199) [Cummings *et al.* 2008]. Studies of the general practice population puts the incidence of Crohn's disease at 8.3 cases per 100,000 people annually, and the prevalence at 144.8 cases per 100,000 people [Rubin *et al.* 2000].

1.2.2 Sex, age and racial incidence

The onset of Crohn's disease has a bimodal distribution. The first and largest peak occurs between the ages of 15-30 years [Rubin *et al.* 2000]; around 10% of cases occur in individuals younger than 18 years of age [Hanauer 2006]. The second, much smaller peak is between 50 - 70 years [Andres *et al.* 1999].

The disease tends to occur in peoples of a higher socioeconomic group [Andres *et al.* 1999; Loftus 2004]. People of Caucasian and Ashkenazic Jewish origin have higher rates of Crohn's disease than individuals of other backgrounds [Loftus 2004]. Whilst the incidence among ethnic or racial minority groups is still lower than that of Caucasians and Ashkenazic Jewish, the gap between the groups has been closing, with increased incidence of Crohn's disease over recent years in African Americans [Loftus 2004] and in second generation South Asians who have migrated to developed countries [Montgomery *et al.* 1999].

1.3 GENETIC FACTORS IMPLICATED IN CROHN'S DISEASE

1.3.1 Family occurrence

Several family association studies have demonstrated a genetic contribution to the pathogenesis of Crohn's disease [Weterman *et al.* 1984; Monsen *et al.* 1991; Orholm *et al.* 1991; Halme *et al.* 2006]. In one such study from Denmark, analysis of 133 Crohn's disease patients revealed that first degree relatives had a 10-fold increased risk for Crohn's disease, after standardization for age and sex, compared to the general population [Orholm *et al.* 1991]. This was subsequently followed up by a much larger study several years later using a national database consisting of millions of patients, where similar outcomes were observed [Orholm *et al.* 1999].

The age at which Crohn's disease occurs also seems to be influenced genetically - patients with Crohn's disease and a family history tend to get their disease at an earlier age than patients without a family history [Van Kruiningen *et al.* 2007].

In addition, twin studies have revealed that monozygotic twins with Crohn's disease have a much higher disease concondance (50%) of the disease than dizygotic twins (3.8%), indicating that genetic factors play an important role in Crohn's disease development [Halfvarson *et al.* 2003]. Similar findings have been observed from other independent groups [Burak *et al.* 1998; Orholm *et al.* 2000; Halme *et al.* 2006; Spehlmann *et al.* 2008].

1.3.2 The immune system

The immune system can be divided into two systems, namely the innate (non-specific) and the adaptive (specific), which operate at both the local (humoral) and systemic level [Wood, 2001]. The innate and adaptive immune systems work together through direct cell contact and through interactions involving chemical mediators, cytokines and chemokines. Aberrant functioning of the immune system has been implicated in Crohn's disease [van Lierop *et al.* 2009; Gersemann *et al.* 2009].

The epithelium within the intestine acts as the interface between the intestinal lumen and the intestinal tissue; it consists of a single layer of cells protected by a layer of mucus (produced by goblet cells) on the luminal surface [Macpherson *et al.* 2004]. Paneth cells within the epithelium produce antimicrobial peptides such as α-defensins [Ayabe *et al.* 2000], lysozyme [Chung *et al.* 1988; Cross *et al.* 1988] and phospholipase A2 [Talvinen *et al.* 2001; Keshav 2006], all of which act as a first line defence against pathogens, and serve as part of the innate immune response. If Paneth cells come into direct contact with invading microbes or microbial molecules, production of further and higher amounts of antimicrobial peptides is induced [Ayabe *et al.* 2000]. The released antimicrobial peptides have the ability to attract antigen-presenting cells and lymphocytes [Yang *et al.* 2002], in this way, Paneth cells

play a role in both the innate immune response, and in activating the adaptive immune system.

In healthy individuals, Paneth cells are found only in the small intestine, in Crohn's disease, Paneth cells have been found to also develop in the colonic mucosa [Müller *et al.* 2005]. Altered antimicrobial peptide production by Paneth cells has been implicated in Crohn's disease by several independent groups [Lala *at el.* 2003; Wehkamp *at el.* 2005; Wang *at el.* 2007].

Innate immunity is often considered a first line defence against microbial infection; it serves to protect the host from infection by other organisms, in a non-specific manner, without any long lasting specific immunity [Medzhitov, 2007; Wood, 2001]. The innate immune response involves several host cells and components, including epithelial barriers, phagocytes (neutrophils, and monocytes/macrophages), mast cells, Paneth cells, natural killer cells, the complement system, and innate immunity associated cytokines. Together these cells act to recognize and eliminate foreign antigens through phagocytosis. In Crohn's disease, this process is understood to be deregulated, and several genes have been identified which are thought to be involved in this process and which are closely linked to Crohn's disease susceptibility [The Wellcome Trust Case Control Consortium, 2007; Barrett *et al.* 2008; Fisher *et al.* 2008].

Conversely, the adaptive immune is triggered by the innate immune system and acts to recognize and remember specific pathogens, conferring long lasting specific immunity. Cells involved in the adaptive immune response involve B cell lymphocytes (which secrete antibodies) and memory T cells (also a lymphocyte cell). B cells mature mainly under the influence of bone marrow and give rise to lymphoid populations which, on contact with

antigen, proliferate and differentiate into plasma cells. In turn, these plasma cells produce a humoural factor (an antibody) which is specific for the antigen and which is able to neutralise or eliminate the antigen. T lymphocytes mature under the influence of the thymus, and upon stimulation by antigen, give rise to cellular immunity – this provides the immune system with a memory of the antigen after the infection has subsided) [Wood, 2001].

1.3.3 Susceptibility genes

The pathogenic mechanisms of Crohn's disease are poorly understood. Fine mapping as well as candidate gene studies have led to the identification of a number of susceptibility genes for Crohn's disease. A bioinformatics based approach has led to the identification of upwards of 30 genes which have been implicated in the pathogenesis of Crohn's disease [The Wellcome Trust Case Control Consortium, 2007; Barrett *et al.* 2008; Fisher *et al.* 2008].

Well documented genes thought to be associated with Crohn's disease include autophagy genes *ATG16L1* (ATG16 autophagy-related 16-like 1) and *IRGM* (immunity-related guanosine triphosphatase). Other genes identified included *NOD2/CARD15* (nucleotide-binding oligomerization domain containing 2 / caspase recruitment domain family, member 15), *MST-1* (macrophage stimulating 1, a protein that influences motile activity and phagocytosis by peritoneal macrophages) and interleukin-23 receptor (*IL23R*).

Recent work by Barrett *et al.* has identified several other gene loci to be associated with Crohn's disease [Barrett *et al.* 2008]. These newly identified loci contain genes such as *PTPN2 and PTPN22* (protein tyrosine phosphate, nonreceptor types 2 and 22), *LRRK2* (leucine-rich repeat kinase 2, a multi-

domain protein expressed mainly in the cytoplasm of myeloid cells and monocytes, and potentially in autophagy [Plowey *et al.* 2008]), *ITLN1* (intelectin-1), *CCR6* (chemokine receptor 6, a member of the G protein–coupled chemokine receptor family), IL12B (interleukin-12 B, this gene encodes a p40 subunit which forms part of both IL-12 and IL-23), *STAT3* (signal transducer and activator of transcription 3) and *JAK2* (Janus kinase 2. The JAK-STAT signalling pathway is involved downstream of cytokine and growth factor signalling from cell surface receptors, it functions to signal to the nucleus to modify transcription of various genes).

The identified genes have been associated with a diverse range of cellular processes, from regulation of the intestinal epithelial barrier, innate immune responses, adaptive immune responses and cellular signalling, suggesting that a dysregulated immune response to commensal intestinal bacteria and possibly defects in mucosal barrier function or bacterial clearance may be involved in Crohn's disease pathogenesis [Sartor 2006].

1.3.3.1 Innate immune response

The gene products of *NOD2/CARD15*, *NOD1* and *TLR4* are involved in the recognition of bacterial products.

In 2001, positional cloning identified *CARD15* (caspase recruitment domain family, member 15) / *NOD2* (nucleotide-binding oligomerization domain containing 2) as the first confirmed Crohn's disease-susceptibility gene [Hugot *et al.* 2001; Ogura *et al.* 2001]. Since then, more than 30 variations in the *NOD2* gene have been associated with an increased risk of Crohn's disease in the lower part of the small intestine (the ileum). This increased risk has been found only in Caucasian (white) populations.

The NOD2 protein is active in immune cells such as monocytes, macrophages, paneth cells, and dendritic cells; it acts as a cytoplasmic sensor of the bacterial peptidoglycan component muramyl dipeptide [Bonen *et al.* 2003; Voss *et al.* 2006]. Once activated, NOD2 activates NF-κB (nuclear factor-kappaB), which in turn regulates the activity of multiple genes, including genes that control immune responses and inflammatory reactions which help protect the intestine from bacterial invasion.

1.3.3.2 Intestinal epithelial barrier function

The NOD2 protein is also active in Paneth cells, found in the base of the crypts, particularly in the distal small intestine [Lala *et al.* 2003]. It can be envisaged that mutations in NOD2 would affect regulation of Paneth cell mediated responses against intestinal bacteria, and perhaps the intestinal barrier itself [Ogura *et al.* 2003].

The gene products of *MDR1* (multidrug resistance gene), *OCTN* (organic carion transporters) [Reinhard *et al.* 2006] and *DLG* (discs large homolog) are thought to play a role in maintaining the intestinal epithelial barrier [Van Limbergen *et al.* 2007]. All have gene mutations which have been associated with Crohn's disease.

The *MDR1* gene encodes an efflux pump of amphipathic toxins, it is highly expressed in distal small bowel and colon - genetic variants in *MDR1* could interfere with mucosal detoxification [Satsangi *et al.* 1996; van Heel *et al.* 2004]. Under specific pathogen-free conditions, MDR-1 knockout mice are susceptible to the development of a severe, spontaneous intestinal inflammation that is preventable by, and treatable with, antibiotics [Panwala *et al.* 1998].

DLG5 is a member of the caspase recruitment domain (CARD) interaction network family [Friedrichs *et al.* 2008] and of the membrane-associated guanylate kinase (MAGUK) family [Van Limbergen *et al.* 2007]. MAGUKs are known to form scaffolds for proteins involved in intraceullar signal transduction. *DLG5* encodes a scaffolding protein involved in the maintenance of epithelial integrity [Stoll *et al.* 2004]; variants in DLG5 protein could therefore interfere with the epithelial barrier function.

1.3.3.3 Cellular signalling

A recent genome wide study [The Wellcome Trust Case Control Consortium, 2007] found a highly significant association between Crohn's disease and the *IL23R* gene on chromosome 1p31. This gene encodes a subunit of the receptor for the proinflammatory cytokine interleukin-23 [Duerr *et al.* 2006], which is pivotal in the differentiation of T helper (Th17) cells. In animal models, the Th17 T cell subset mediates chronic and autoimmune inflammatory conditions [Van Limbergen *et al.* 2007]. Variants of this gene can confer an increased risk of Crohn's disease development [Amre *et al.* 2008; Taylor *et al.* 2008], whilst a relatively rare variant has been found to confer strong protection against developing Crohn's disease [Dubinsky *et al.* 2007].

1.3.3.4 Autophagy

Significant associations between an intergenic region on 10q21.1 and a coding variant in *ATG16L1* (ATG16 autophagy related 16-like 1) have been identified for Crohn's disease [Hampe *et al.* 2007; Rioux *et al.* 2007]. *ATG16L1* is highly expressed in intestinal epithelial and immune cells and is important for autophagy, a cellular process involved in the degradation and processing of bacteria [Hampe *et al.* 2007; Rioux *et al.* 2007]. Rioux *et al.* demonstrated that *ATG16L1* was expressed in intestinal epithelial cell lines, and knockdown

of the gene abrogates autophagy of *Salmonella* Typhimurium, suggesting that autophagy and host cell responses to intracellular microbes are involved in the pathogenesis of Crohn's disease [Rioux *et al.* 2007].

Several variations in or near the *IRGM* gene (5q33.1) have been associated with an increased risk of developing Crohn's disease. Variants in *IRGM* (immunity-related GTPase family, M), encoding a GTP-binding protein which induces autophagy, has also been shown to be associated with Crohn's disease [The Wellcome Trust Case Control Consortium, 2007]. The product of this gene is involved in elimination of intracellular bacteria, including *Mycobacterium tuberculosis* [Singh *et al.* 2006]. Reduced function and/or activity of this gene would be expected to prevent the immune system from destroying harmful bacteria effectively leading to persistence of intracellular bacteria. An abnormal immune response to bacteria in the intestinal walls may lead to chronic inflammation and the digestive problems characteristic of Crohn's disease.

1.4 EVIDENCE FOR BACTERIAL INVOLVEMENT IN CROHN'S DISEASE

1.4.1 The role of the faecal stream

Some of the clearest evidence to support a role for bacterial involvement in Crohn's disease comes from observations following surgery; the creation of a diverting ileostomy prevents postoperative recurrence, but disease often recurs rapidly following reanastomosis [Rutgeerts *et al.* 1991]. Fractionation of the faecal stream followed by selective reintroduction demonstrated exacerbation of inflammation by particles greater than 0.22 μm – consistent with the involvement of a bacterial factor [Harper *et al.* 1985].

1.4.2 Stool microbiology

1.4.2.1 Gut microbiota In the healthy individual

The normal human colonic microbiota is made up of a complex ecosystem of over 500 species of bacteria [Madsen *et al.* 1999; Prindiville *et al.* 2000]. These bacterial species comprise 95% of the cells in the human body [Dunne *et al.* 1999]. There is a gradual increase in the population along the small bowel, from approximately 10^4 colony forming units (CFU) per gram of luminal content in the jejunum to 10^7 in the distal ileum, with a predominance of Gram negative aerobes and some obligate anaerobes [Guarner *et al.* 2003]. Conversely, the human colon is heavily populated with anaerobes with bacterial counts in the region of 10^{14} per gram of luminal content [Egert *et al.* 2006].

Traditionally, culture methods together with phylogenetic classification have been used to identify and characterise faecal microbiota, but with the limitation that around 70 – 90% of bacteria are, to date, unculturable [Eckburg *et al.* 2005]. More modern methods have allowed for characterisation of 16S rRNA sequence diversity [Rinttila *et al.* 2004] and the use of denaturing gradient gel electrophoresis in combination with PCR [Bibiloni *et al.* 2005] have allowed for insight and detection of the normal microflora, and changes in response to disease [Andersson *et al.* 2008].

In the healthy gut, Firmicutes (low-G+C Gram positives), *Bacteroides* and Actinobacteria (high-G+C Gram positives) are the most common species [Backhed *et al.* 2005]. Probably the single most common is *Bacteroides* spp., which account for the majority of intestinal obligate anaerobes in the colon with counts of between 10^{10} and 10^{11} cells per gram of intestinal contents. *E. coli* is the predominant facultative anaerobe of the human colonic microbiota

[Boudeau *et al.* 1999] but accounts for less than 0.7% of the total human microbiota.

1.4.2.2 Gut microbiata alterations in Crohn's disease

Sokol *et al.* used fluorescent *in situ* hybridization adapted to flow cytometry to demonstrate that the bacterial composition of fecal samples from 13 patients with active Crohn's disease had significantly lower levels of *Clostridium leptum* compared to healthy subjects [Sokol *et al.* 2006], whilst Manichanh *et al.* demonstrated a significant reduction in the Firmicutes phylum in Crohn's disease compared to healthy control patients [Manichanh *et al.* 2006].

1.4.3 Role of Mycobacterium avium subsp paratuberculosis (MAP)

In 1913, Dalziel, a Scottish physician, likened the clinical appearance of chronic intestinal enteritis in man (later defined as Crohn's disease) with the appearance of Johne's disease in cattle (paratuberculosis) as - 'the histological characters and naked-eye appearances are as similar [in cattle] as may be to those we have found in man' [Dalziel 1989]. This suggested for the first time the idea that Crohn's disease might be caused by a specific organism, like Johne's disease, which is caused by *Mycobacterium avium* subspecies *paratuberculosis* (MAP). Since then, others have noted the clinical and pathological similarities between Johne's and Crohn's disease [Chiodini 1989; Greenstein 2003].

Since then however, the literature has been divided on whether MAP is a causative agent in Crohn's disease, with several dozen studies providing evidence to either prove or disprove the association of MAP with Crohn's disease [Sartor 2005]. Whilst MAP has been detected in Crohn's disease

tissue by culture, PCR and by FISH analysis by some groups [Chiodini *et al.* 1984; Sanderson *et al.* 1992; Hulten *et al.* 2001; Sechi *et al.* 2001; Autschbach *et al.* 2005], not all groups have been able to detect the presence of MAP with reported detection rates ranging from 0 – 100% in Crohn's disease tissue [Mishina *et al.* 1996; Behr *et al.* 2006]. However, MAP has been cultured from the blood of Crohn's disease patients [Naser *et al.* 2004] and has been found to elicit increased serological responses in Crohn's disease patients [Olsen *et al.* 2001; Naser *et al.* 2004].

MAP is known to persist in the food chain in both meat and milk [Millar *et al.* 1996; Mishina *et al.* 1996; Corti *et al.* 2002; Grant *et al.* 2002], so a ready source of potential infection exists. Despite this, there is a lack of epidemiological data supporting transmissible infection of MAP in Crohn's disease [Abubakar *et al.* 2007], disease status is not exacerbated by immunosuppressive agents [Sartor 2005], and there is no therapeutic response to traditional antimycobacterial antibiotics [Thomas *et al.* 1998]. A large scale meta-analysis by Feller *et al.* did find an association between MAP and Crohn's disease, but it was found that the 'role [of MAP] in the aetiology of Crohn's disease remains to be defined' [Feller *et al.* 2007].

1.4.3 Mucosal *E. coli* in Crohn's disease

Schultsz *et al.* detected *E. coli* adherent to the rectal mucosa in Crohn's disease which possessed adherence factors, but did not detect any differences in frequency of *E. coli* in either control or diseased samples [Schultsz *et al.* 1997]. Subsequently the same group, by use of *in situ* hybridisation, demonstrated a marked increase in bacterial 16S ribosomal DNA within the overlying mucus of diseased rectal mucosal biopsies compared to controls [Schultsz *et al.* 1999].

Darfeuille-Michaud *et al.* demonstrated that early and chronic ileal lesions of Crohn's disease are often colonised by mucosally-associated *E. coli* strains devoid of the virulence genes that are generally associated with enteropathogenic strains of *E. coli* [Darfeuille-Michaud *et al.* 1998]. Since then, nine independent studies have revealed an increased presence of mucosa-associated *E. coli* in Crohn's disease [Darfeuille-Michaud *et al.* 2004; Martin *et al.* 2004; Ryan *et al.* 2004; Mylonaki *et al.* 2005; Swidsinski *et al.* 2005; 2006; Baumgart *et al.* 2007; Kotlowski *et al.* 2007; Sasaki *et al.* 2007]. This group of *E. coli* have been designated as a new pathotype, termed adherent-invasive *E. coli* (AIEC) [Darfeuille-Michaud 2002].

1.4.3.1 AIEC characteristics

AIEC do not possess any of the known genetic invasive determinants described for enteroinvasive (EIEC) or enteropathogenic *E. coli* (EPEC) and *Shigella* strains [Darfeuille-Michaud 2002]. Despite this, they have been shown to both adhere and invade into several *in vitro* intestinal cell lines [Darfeuille-Michaud *et al.* 2004; Martin *et al.* 2004] although there is little or no direct evidence for their presence within normal human intestinal epithelial cells in human tissue whereas they can be found within tissue macrophages [Liu *et al.* 1995; Ryan *et al.* 2004].

The AIEC have been shown to stimulate the release of the pro-inflammatory chemokine interleukin-8 (IL-8) from epithelial cells *in vitro* [Martin *et al.* 2004; Eaves-Pyles *et al.* 2008; Subramanian *et al.* 2008]. The AIEC preferentially belong to phylogenic groups B2 and D that are associated with uropathic, avian and extraintestinal virulent *E. coli* strains (Uropathogenic (UPEC), avian pathogenic (APEC) and Extraintestinal pathogenic (ExPEC)) [Kotlowski *et al.* 2007]. In addition to their association with human Crohn's disease, these

AIEC have been shown to be associated with granulomatous colitis in boxer dogs [Simpson *et al.* 2006].

One ileal isolate, LF82 has been investigated in detail; it has been reported to replicate extensively within mature phagolysosomes of macrophages [Bringer *et al.* 2006]. It has been found that deletion of the genes *yfgL* and *Nlpl* (both of which encode lipoproteins) from *E. coli* LF82 lead to a decrease in invasive ability of LF82 [Barnich *et al.* 2004; Rolhion *et al.* 2005]. LF82 has also been shown to adhere to primary ileal epithelial cells isolated from Crohn's disease patients. The adherence was dependent on type 1 pili expression on the bacterial surface and on carcinoembryonic antigen–related cell adhesion molecule 6 (CEACAM6) expression on the apical surface of ileal epithelial cells [Barnich *et al.* 2007], it was found that the process could be blocked by 2% D-mannose.

It should be noted that the demonstration of the presence of AIEC within diseased tissue does not automatically suggest a pathogenic role for these organisms. Other factors may be involved in promoting mucosal dysbiosis, such as glycosylation changes [Rhodes 1996], overexpression of fibronectin [Isenmann *et al.* 2002] and carcinoembryonic antigen expression [Greenstein *et al.* 1982], which may promote recruitment of bacteria including *E. coli* to the mucosa.

1.4.4 Intra-mucosal bacteria in Crohn's disease

E. coli, Listeria, and Group F Streptococci have been demonstrated within macrophages in Crohn's disease tissue [Liu *et al.* 1995]; however, not all groups have been able to identify *Listeria* in Crohn's disease patients through either PCR detection [Chiba *et al.* 1998; Chen *et al.* 2000] or

immunohistochemistry [Walmsley *et al.* 1998]. Walmsley *et al.* however were able to observe *E. coli* on the luminal surface of epithelium and in ulcers in 35% of Crohn's disease patients [Walmsley *et al.* 1998].

1.4.5 Other bacterial species identified in Crohn's disease

Tiveljung *et al.* in 1999 demonstrated by *in situ* hybridisation, an increase in *Helicobacter* spp.-, *Mycobacterium paratuberculosis*-, *Listeria monocytogenes*- and *E. coli*-like 16S rDNA sequences in Crohn's disease compared with control biopsies [Tiveljung *et al.* 1999].

1.4.6 Role of the adaptive immune response to bacterial antigens in Crohn's disease pathogenesis

Crohn's disease has been associated with elevated adaptive immune responses to gut microbiota. Crohn's disease patients are known to have antibodies against *E. coli* outer-membrane porin C (OmpC), *Pseudomonas fluorescens*-associated sequence I2, bacterial flagellin and *Saccharomyces cerevisiae* (ASCA) [Landers *et al.* 2002], indicating a loss of host-tolerence against these bacterial species and products. The presence of anti-OmpC and anti-I2 antibodies has been shown to be associated with a complicated disease pattern amongst Crohn's disease patients [Arnott *et al.* 2004].

Marks *et al.* reported a substantially diminished IL-8 response by peripheral blood monocyte-derived macrophages from Crohn's disease patients to pro-inflammatory stimuli [Marks *et al.* 2006]. It was found that the defective IL-8 response was associated with a reduced recruitment of neutrophils to sites of mucosal injury (the biopsy site), and the defect in IL-8 response was absent in healthy controls. It has also been observed that macrophages from smokers

(who have a higher risk of developing Crohn's disease) lack the ability to kill *Legionella pneumophila* [Matsunaga *et al.* 2001]. Diseases such as the inherited conditions chronic granulomatous disease and glycogen storage disease type 1b are associated with a Crohn's disease like intestinal disease; both these conditions are well characterised as having defects in phagocyte killing of bacteria [Isaacs *et al.* 1985; Sanderson *et al.* 1991].

1.4.7 Animal models

Several animal models exist which replicate to some extent the human phenotype of Crohn's disease, and which indicate the importance of gut microbiota for the disease [Wirtz *et al.* 2007]. These mouse models typically have defects in epithelial integrity/permeability, or defects in the innate or adaptive immune system.

Several Crohn's disease associated genes, such as OCTN1, OCTN2, DLG5, NOD2/CARD15 and ATG16L1 have been shown to be related to the biological function of epithelial cells, and mutations in these genes could potentially lead to increased intestinal permeability.

An interesting mouse model which provides direct evidence for the importance of an intact epithelial barrier is that of transgenic/chimeric mice expressing a dominant negative mutant form of the cell adhesion molecule N-cadherin, along the crypt villus axis [Hermiston *et al.* 1995]; these mice develop a chronic inflammatory bowel disease in areas of chimeric/leaky epithelium.

In addition, missense mutations in the gene for keratin 8, a major intermediate filament protein present in intestinal enterocytes, have been

identified in a subset of patients with Crohn's disease [Owens *et al.* 2004]. Mice deficient for keratin 8 develop colonic hyperplasia and colitis as a result of epithelial rather than immune cells defects [Baribault *et al.* 1994]. Treatment with antibiotics markedly decreased intestinal inflammation, implying that luminal bacteria play a role in Crohn's disease [Baribault *et al.* 1994].

The *multidrug resistance (MDR)* gene 1, encoding P-glycoprotein 170, is responsible for drug resistance to chemotherapy in certain types of cancer, is expressed in the intestinal epithelium and subsets of hematopoietic cells [Ambudkar *et al.* 2003]. *Mdr1*$\alpha^{-/-}$ mice display spontaneous bowel inflammation triggered by the bacterial microbiota [Panwala *et al.* 1998].

SAMP1/YitFc (senescence-accelerated mouse - Samp) mice develop spontaneous inflammation in the terminal ileum [Matsumoto *et al.* 1998], a common site of inflammation in Crohn's disease. Germ-free housed SAMP mice develop intestinal inflammation spontaneously; however they do so to a lesser extent than when housed in conventional conditions, implying that both epithelial permeability and the microflora are important factors in inflammation.

Several mouse models implicate a defective innate immune system in association with aberrant inflammation. Innate immune cells recognise foreign organisms through patter recognition receptors (PRRs) which recognise pathogen-associated molecular patterns (PAMPs). PRRs include Toll like receptos (TLR), nucleotide-binding oligomerization domain-like (NOD-like) receptors (NLRs) and C-type lectin receptors (CLRs) [Akira *et al.* 2004; Inohara *et al.* 2003]. Generally, the interaction between PPRs and PAMPs

results in the activation of innate immune cells such as dendritic cells and macrophages which function to eliminate the foreign organism.

Mice with a mutant STAT3 gene (signal transducter and activator of transcription 3) in macrophages and neutrophils develop a spontaneous enterocolitis [Takeda *et al.* 1999]. When crossed with TLR4−/− or MyD88−/− mice the double deficient mice developed significantly less colitis, indicating the critical requirement of the bacterial microflora [Kobayashi *et al.* 2003].

STAT 3 is also involved in the signal transduction pathway of Interleukin-10 (IL-10). Interleukin-10 (IL-10) is an anti-inflammatory cytokine. IL-10 gene-deficient mice develop a patchy chronic enterocolitis with massive infiltration of lymphocytes, activated macrophages, and neutrophils [Kuhn *et al.* 1993]. This colitis develops when the mice are reared in conventional animal care facilities; conversely, they do not develop colitis when reared in germ-free conditions, again suggesting that luminal bacteria are a significant factor in the onset and chronicity of inflammation [Madsen *et al.* 1999]. These mice demonstrate significant alterations in the species and the levels of bacteria colonizing the colon, suggesting that genetic factors in the host may be critical in controlling bacterial colonization [Madsen 2001].

Further studies in this mouse model have revealed that increased mucosal adherence in the colon precedes the development of colitis, and that this increase is coupled with a reduction of colonic luminal *Lactobacillus* spp.. Restoring the levels of *Lactobacillus* spp. to those of the normal mouse colon resulted in a reduced concentration of adherent bacteria, a reduction in bacteria translocated across the epithelial cell layer, and attenuation of the colitis [Madsen *et al.* 1999; Madsen *et al.* 1999; Schultz *et al.* 2000].

1.5 ENVIRONMENTAL FACTORS IMPLICATED IN CROHN'S DISEASE

1.5.1 General observations

Several lines of evidence implicate environmental factors as risk factors or association with Crohn's disease development. Factors such as diet, smoking, occupation and wealth all appear to have a connection to Crohn's disease development.

Poor sanitary conditions and exposure to infection appear to protect against Crohn's disease, in common with diseases such as multiple sclerosis, rheumatoid arthritis and asthma. Children who have been raised in more spacious conditions have a higher risk of developing Crohn's disease than those raised in more cramped conditions [Krishnan *et al.* 2002] whilst people in higher socioeconomic groups have a higher risk of Crohn's disease development [Krishnan *et al.* 2002]. Additionally, Crohn's disease is more common in 'white-collar' professionals in sedentary indoors jobs compared with 'blue-collar' professionals who work outside [Sonnenberg 1990].

In addition, there has been clear emergence of Crohn's disease in the economically developed nations of Western Europe and North America during the past century, whilst incidence remains low in less economically developed countries [Calkins *et al.* 1986]. Dramatic increases in incidence observed during the 1950s and 1960s [Miller *et al.* 1974] and during the remainder of the 20th century in developed countries support the theory that environmental factors may contribute substantially to Crohn's disease development [Krishnan *et al.* 2002].

1.5.2 Dietary factors

There are several lines of evidence which suggest that the diet may play an important role in the development and prevalence of Crohn's disease. Malnutrition is known to worsen the course of disease progression in Crohn's disease, since both macronutrient and micronutrient deficiencies lead to impaired inflammatory and immune intestinal response [Sanderson *et al.* 2005]. There is good evidence supporting the benefits of enteral nutritional feeding with elemental or whole protein feed as either primary or adjuvant therapy for Crohn's disease [Ricour *et al.* 1977; Morin *et al.* 1982]. Several studies have attributed these benefits in part to a decrease in systemic inflammatory markers as a consequence of enteral feed [O'Morain *et al.* 1984; Thomas *et al.* 1993; Akobeng *et al.* 2007]. Others report that enteral feeding directly affects intestinal inflammation by regulating gene expression in the gut epithelium [Sanderson *et al.* 2005; de Jong *et al.* 2007], as well as modulating intestinal inflammatory and immune mediators production [Fell 2005; Genton *et al.* 2005; Sanderson *et al.* 2005; Menezes *et al.* 2006; de Jong *et al.* 2007; Yamamoto *et al.* 2007]. In some studies however, the use of enteral feeding was less effective than conventional therapies such as steroids or aminosalicylates [Zachos *et al.* 2007]. The consumption of fruits, vegetables and dietary fibre has been found to be protective against Crohn's disease development [Amre *et al.* 2007].

Increased intake of dietary sugar has long been linked as a risk factor for increased occurrence of Crohn's disease [Martini *et al.* 1976; Miller *et al.* 1976; Thornton *et al.* 1979; Mayberry *et al.* 1980; Silkoff *et al.* 1980; Katschinski *et al.* 1988]. There is also evidence to suggest that frequent fast-food intake confers a 3- to 4-fold greater risk for developing Crohn's disease [Persson *et al.* 1992], whilst consumption of long-chain omega-3 fatty acids

has been reported to be negatively associated with Crohn's disease [Amre *et al.* 2007].

There has been a rapid recent rise in Crohn's disease in Japan [Yao *et al.* 2000] which has paralled the introduction and uptake of a western style diet and an increase in animal fat consumption there [Shoda *et al.* 1996; Sakamoto *et al.* 2005]. Indeed, it has been observed that an increase in dietary fat intake can counteract the benefits obtained from an elemental diet in the treatment of Crohn's disease in Japan [Bamba *et al.* 2003].

Additionally, there has been a relatively recent increase in Crohn's disease development amongst migrants to 'First-world' countries [Montgomery *et al.* 1999], it is possible that this may in part be due to a change of diet in the new home country.

1.5.3 Smoking

Probably the most convincing example of the influence of the environment upon the development of Crohn's disease is that of smoking tobacco. In 1984, a case-control study by Somerville *et al.* reported that the relative risk of developing Crohn's disease was 4.8 for those who smoked before disease onset, and 3.5 for those with a current smoking habit [Somerville *et al.* 1984]. In other studies, it has been found that smoking tobacco increases the need and frequency of surgery [Lindberg *et al.* 1992; Breuer-Katschinski *et al.* 1996], the frequency of progression to complicated disease (defined by development of strictures or fistulae [Mahid *et al.* 2007]), penetrating intestinal complications [Lindberg *et al.* 1992; Louis *et al.* 2003; Picco *et al.* 2003], and a higher relapse rate [Cosnes *et al.* 1999; Lakatos *et al.* 2005], whilst cessation of smoking improves the disease course [Thomas *et al.*

1998]. Smoking is known to affect both systemic and mucosal immunity, affecting both innate and immune functions, such as altering the ratio of T-helper to T-suppressor cells [Sopori 2002] and affecting the ability of macrophages to kill bacteria [King *et al.* 1988; Matsunaga *et al.* 2001].

1.6 PATHOPHYSIOLOGY OF CROHN'S DISEASE

1.6.1 Initial presentation

There is controversy surrounding the site of the initial lesion in Crohn's disease. It has been suggested that it may be the granuloma, which can be found within the mucosa despite entirely normal overlying epithelium [Makiyama *et al.* 1984; Wakefield *et al.* 1991]. Conversely, there is a growing body of evidence suggesting that the initial lesions of Crohn's disease are in fact the aphthous lesions.

Lockhart-Mummery and Morson [Lockhart-Mummery *et al.* 1960; Morson 1972] reported as far back as the 1960s that an early microscopic change in Crohn's disease was ulceration of lymphoid follicles and Peyer's patches in the terminal ileum. These ulcerations have subsequently been termed aphthous lesions, after the Greek word *aptha*, roughly translated as an inflamed spot [Kinjo *et al.* 2004]. These aphthous lesions under endoscopic examination appear as superficial ulcers, 1–5 mm in diameter, with surrounding erythema. Aphthoid lesions can be found in 70% of Crohn's disease patients [Rickert *et al.* 1980], and have been described as as early endoscopic signs of Crohn's disease recurrence in the neoterminal ileum following ileocolonic anastomosis [Rutgeerts *et al.* 1984].

In Crohn's disease of the large bowel, aphthoid lesions frequently occur over lymphoid aggregates [Fujimura *et al.* 1996], whilst in Crohn's disease of the small bowel, these lesions typically occur over Peyer's patches [Rickert *et al.* 1980; Rutgeerts *et al.* 1984; Olaison *et al.* 1992] which are structured collections of lymphoid follicles [Van Kruiningen *et al.* 2002]. Interestingly, Van Kruiningen *et al.* noted that there is an age related increase in the frequency of Peyer's patches, which correlates well with the increase in Crohn's disease with age [Van Kruiningen *et al.* 1997].

As Crohn's disease progresses, it is thought that the aphthous lesions enlarge to form stellate ulcers and ultimately, deeper longitudinal and transverse linear ulcers [Hizawa *et al.* 1994]. Intervening regions of mucosa not affected by ulceration can create the typical cobblestone appearance characteristic of Crohn's disease. These findings have been confirmed by endoscopic and radiographic studies in the small intestine [Olaison *et al.* 1992; Matsumoto *et al.* 2000], but are less obvious in colonic Crohn's disease [Ni *et al.* 1986].

1.6.3 M-cells and bacterial sampling of the lumen

Since the initial lesions which are often observed in Crohn's disease occur in the region of lymphoid aggregates in the large bowel, and Peyer's patches in the small bowel, it is possible that M-cells present in the 'dome' epithelia that overlies Peyer's patches and lymphoid follicles might play an important role in the pathophysiology of Crohn's disease.

1.6.3.1 Peyer's patches

Peyer's patches are named after the 17th-century Swiss anatomist Hans Conrad Peyer and are located along the antimesenteric side of the small intestine [Corr *et al.* 2008]. The Peyer's patches consists of the Peyer's patch

lymphoid follicle (containing B- and T- lymphocytes), follicular dendritic cells and macrophages; overlying the Peyer's patches is the follicle-associated epithelium (FAE) [Neutra *et al.* 2001], a one-cell-thick layer composed of enterocytes and specialized epithelial cells termed M-cells [Owen *et al.* 1974]. The FAE forms the interface between the intestinal lymphoid system and the intestinal luminal environment.

1.6.3.2 M-cells - origin

The origin of M-cells within the FAE is unclear and is the subject of much debate. Intestinal epithelial cells in the FAE originate from stem cells in the crypts located between villi and the dome epithelium – within the crypts, there are two distinct axes of migration and differentiation [Heath 1996]. Cells on the villous side of the crypt migrate upwards to form the villi, differentiating into absorptive enterocytes, goblet cells and endocrine cells as they migrate upwards [Sierro *et al.* 2000], where upon reaching the tip of the villus, they are shed into the intestinal lumen [Sierro *et al.* 2000; Kiesslich *et al.* 2007]. Cells on the FAE side of the crypt migrate into the dome epithelia, where they differentiate into absorptive enterocytes and M-cells [Gavrieli *et al.* 1992].

It is not known if cells within the crypt commit to become M-cells early on, or if the formation of M-cells occurs as a result of transformation from existing enterocytes within the dome FAE [Nicoletti 2000]. A well established *in vitro* M-cell culture system demonstrates that enteroyctes can be converted into M-cells by murine Peyer's patch lymphocytes [Kerneis *et al.* 1997] or Raji B lymphocyte cells [Gullberg *et al.* 2000; des Rieux *et al.* 2007]. However, since this model utilises adenocarcinoma cells (Caco2) that do not behave like normal enterocytes, it is difficult to know if this *in vitro* M-cell model is a true indication for the *in vivo* situation. Indeed, one group has reported that B

cells are not absolutely required for the generation of M-cells from Caco2 cells – they found the presence of M-like cells in lymphocyte untreated Caco-2 monolayers [Blanco *et al.* 2006].

Gerbert *et al.* demonstrated the presence specialised dome epithelia associated crypts which differed from ordinary crypts in size, shape, cellular composition and location. Within these specialised crypts, Gerbert *et al.* was able to identify the presence M-cell precursor cells using histological and ultrastructural techniques, indicating that *in vivo* M-cells arise from a distinct cell lineage [Gebert *et al.* 1999].

It has also been shown that M-cells numbers increase in response to microbial challenge. Smith *et al.* showed that in mouse FAE, the number of M-cells present increased upon transfer of germ-free mice into conventional housing [Smith *et al.* 1987], with several *in vitro* studies (using mouse or rabbit biopsies) confirming these findings [Savidge *et al.* 1991; Borghesi *et al.* 1996; Borghesi *et al.* 1999]. In contrast, Gebert *et al.* show that increased uptake of particles after inoculation with bacteria depended on increased transport capacity in M cells already present in the FAE, and not upon the formation of new M-cells [Gebert *et al.* 2004].

It should be noted that whilst much of the literature suggests M-cells are only present within the FAE, there is a growing body of evidence reporting the presence of M-cells within villus epithelial cells and cryptopatches in mice [Iwatsuki *et al.* 2002; Jang *et al.* 2004] and in villus epithelial cells in non-human primates [Takahashi *et al.* 2008].

Interestingly, villus M-cells within mice were increased following cholera toxin treatment, implying generation of the M-cell phenotype in response to

potentially pathogenic challenge [Terahara *et al.* 2008], whist in the Rhesus Macaques monkey, oral poliovirus was spatiotemporally internalized into villous M-like cells and engulfed by macrophage-like cells in the lamina propria.

1.6.3.3 M-cells - structure

M-cells are structurally distinct from the surrounding intestinal enterocytes, At the apical surface, M-cells have a poorly organized brush border with short irregular microvilli, in contrast to enterocytes which have a highly organized brush border, with uniform densely packed microvilli [Kerneis *et al.* 1997]. M-cells also lack the thick glycocalyx which is common on surrounding enterocytes; it is thought that this provides the M-cell with greater access to antigens in the gut lumen. The dome epithelium also lacks goblet cells, thus Peyer's patches and lymphoid follicles have little or no overlying mucus layer, allowing the M-cell greater access to antigens in the gut lumen.

1.6.3.4 M-cell markers

Positive markers of M-cells in mice are *Ulex europaeus* 1 (UEA-1) and *Psophocarpus tetragonolobus* (winged bean; WBA) lectins [Clark *et al.* 1993; Giannasca *et al.* 1994], conversely, there are a lack of true definite positive markers for human M-cells. In order to identify markers of human M-cells, recent work has utilized microarray analysis of Caco-2 cells differentiated to M-like cells and established, via microarrary technology, that galectin 9 is apically expressed on M-cells and has promise as an M-cell marker [Pielage *et al.* 2007]. In addition, monoclonal antibodies specific against single carbohydrate epitopes have also been used to identify M-cells in humans [Neutra 1999]. It has also been shown that M-cells express sialyl Lewis A antigen on their apical and subcellular membranes [Giannasca *et al.* 1999], although these results could not be reproduced [Wong *et al.* 2003; Keita *et al.*

2006]. Human M-cells are known to lack certain enterocyte apical surface glycoproteins, such as alkaline phosphatase and sucrase-isomaltase activity, which are typical to the brushborder of enterocytes, and both have been used as negative markers for M-cells [Gebert *et al.* 1996].

1.6.3.5 Functional M-cells

M-cells are known to take up and transport luminal antigens, including proteins, viruses, bacteria, small parasites, and microspheres [Ermak *et al.* 1995; Neutra *et al.* 1996; Gebert *et al.* 1996; Wolf *et al.* 1984].

Antigens are taken up at the M-cell apical surface and are internalised into endosomal tubules and vesicles and large multivesicular bodies that lie between the apical membrane and the intraepithelial pocket (formed from invaginations of the basolateral membrane of the M-cell) [Mestecky *et al.* 2004]. The large vesicles contain the late endosome / lysosome membrane marker lgp20, have an acidic pH [Allan *et al.* 1993] and contain the endosomal protease cathepsin E [Finzi *et al.* 1993]. The presence of acidic endosomal-lysosomal compartments within M-cells, and the fact that M-cells express MHC class II molecules [Allan *et al.* 1993] suggests that M-cells may be involved in antigen processing and presentation.

Following uptake, the antigens are subsequently released from the M-cells into the extracellular 'pocket' region [Mestecky *et al.* 2004], where processing and initiation of immune responses can occur [Kraehenbuhl *et al.* 1992; Neutra *et al.* 1996; Neutra *et al.* 1996]. It is likely that here antigens are captured by immature dendritic cells in the subepithelial dome (SED) regions [Kelsall *et al.* 1996]. Indeed, live attenuated *S. typhimurium* and *Listeria monocytogenes* have been detected in SED dendritic cells [Hopkins *et al.* 2000; Pron *et al.* 2001].

In Peyer's patches and isolated lymphoid follicles, the site of antigen entry into M-cells and subsequent capture by immature dendritic cells occurs in close proximity to organised T-cell and B-cell zones. It has been observed that dendritic cells in the SED are able to deliver antigens to adjacent interfollicular T-cell regions where antigen presentation would be expected to occur [Iwasaki and Kelsall 2000]. It is now generally agreed that oral tolerance is established and maintained at the level of T cells [Strobel *et al.* 1998; Strober *et al.* 1998; Wardrop *et al.* 1999]. Recent studies have identified dendritic cells as key players in the direct or indirect (by way of T cells) induction of oral tolerance [Mowat *et al.* 2003; Nagler-Anderson *et al.* 2001; Viney *et al.* 1998; Weiner *et al.* 2001].

Although M-cells are able to transport luminal antigens [Ermak *et al.* 1995; Neutra *et al.* 1996; Gebert *et al.* 1996; Wolf *et al.* 1984], noninvasive strains of S typhimurium cannot penetrate M cells and are avirulent [Jones *et al.* 1994]. The ability of an antigen to generate an immune response in the gastrointestinal tract could be one of the most critical factors in determining whether mucosal immunity or tolerance is induced. Mucosal tolerance may be the most common immune response because it is necessary to maintain homeostasis. The normal host must readily establish unresponsiveness to commensal bacteria, food antigens, and allergens.

As M-cells are capable of taking up antigen from the lumen and then delivering it to the intestinal lymphoid system, they act as gateway or a portal to the mucosal immune system and this function has been exploited by many invading pathogens. For example, some strains of *Salmonella* Typhimurium have been found to target to M-cells where they cause extensive membrane ruffling and FAE damage [Sansonetti *et al.* 1999], and possible destruction of

the M-cells to perpetuate infection [Jensen *et al.* 1998], whereas pathogens such as *Listeria monocytogenes* and *Shigella flexneri* have been found to be internalized into M cells in a much less disruptive manner [Jensen *et al.* 1998]. M-cells have been also been found to transport *Vibrio cholerae* [Owen *et al.* 1986; Kerneis *et al.* 1997]. The transport of microorganisms through M-cells has been shown to be temperature-dependent, with transport being inhibited at 25°C or lower [des Rieux *et al.* 2005], indicating that transport is an active, not passive process. In addition, M-cells have been shown to transport particulates including latex beads, carbon particles and liposomes and macromolecules including ferritin, horseradish peroxidase, cholera toxin-binding subunit, lectins and antivirus antibodies [Gebert *et al.* 1996].

1.6.3.6 E. coli and M-cells

Several papers have reported *E. coli* adhesion to or translocation through M-cells. Enteropathogenic *E. coli* (EPEC) strain E2348/69 has been found to bind specifically to *in vitro* generated M-cells [Pielage *et al.* 2007], whilst Martinez-Argudo *et al.* reported increased translocation of enteropathogenic *E. coli* (EPEC) across *in vitro* derived M-cells [Martinez-Argudo *et al.* 2007]. Other *E. coli* strains found to adhere to M-cells include *E. coli* 0:124, enteroaggregative (EAEC) and diffuse-adhering *E. coli* (DAEC), enterotoxigenic *E. coli* (ETEC) [Cantey *et al.* 1981]. All of these *E. coli* are classic enteric pathogens.

The use of Ussing chambers provides a useful tool to study *in vitro* human intestinal epithelium [Soderholm *et al.* 1998]. An interesting study by Keita *et al.* utilised the FAE of macroscopically normal ileum from patients with longstanding Crohn's disease and control patients to test the translocation of *E. coli* across the FAE. They observed that the FAE of Crohn's disease patients showed increased *E. coli* K-12 (chemically killed) adhesion following FAE exposure in Ussing chambers, and increased translocation of live *E. coli*

HB101 through cells of the FAE in Crohn's disease [Keita *et al.* 2008]. The paper eloquently demonstrated an increased bacterial load of non-pathogenic *E. coli* within the FAE, raising the possibility that cells within the FAE may also transport AIEC [Gullberg *et al.* 2006].

The FAE has also been implicated as a site of initiation of pro-inflammatory immune responses in rats. One paper suggests that chronic stress, known to affect the course of Crohn's disease, can modulate the FAE of rats. Rats under chronic psychological stress displayed increased *E. coli* K12 passage across FAE, but not across villus epithelium. This implies that stress could lead to increased antigen exposure in Peyer's patches, which may alter proinflammatory immune responses [Velin *et al.* 2004].

1.6.4 Epithelial barrier integrity in Crohn's disease

A tightly regulated epithelial barrier is critical to healthy functioning of the gut. Whilst M-cells provide one route by which this barrier may be breached, recent research suggests another alternative. Epithelial cells of the small intestine migrate to the tip of the villus where they are shed into the lumen, in a process associated with both phosphorylation of myosin light chains (thought to be involved in wound healing) and apoptosis [Bullen *et al.* 2006]. There is a relatively high rate of cell turnover at the tip of the villus, and it has been observed in mice that during this process gaps within the cell layer can be formed. Despite this, the epithelial barrier function is maintained at the apical pole of the epithelial layer [Watson *et al.* 2005]. It has since been observed that these cellular gaps in the epithelial layer also occur in humans [Kiesslich *et al.* 2007]. Whilst it can be assumed that these gaps are a normal component of the intestinal barrier, it does raise the possibility that

dysregulation in this area could provide easy access for luminal microorganisms to the intestinal lymphoid system.

Several groups report a defect in intestinal barrier function to be associated with Crohn's disease. In one study, by Söderholm *et al.* [Soderholm *et al.* 1999], intestinal permeability of Crohn's disease patients together with their healthy first degree relatives and spouses, with healthy unrelated volunteers serving as a control group was investigated. The authors reveal that intestinal permeability is determined by both environmental and genetic factors, and that Crohn's disease patients had higher baseline intestinal permeability than control patients [Soderholm *et al.* 1999]. Work by the same group also revealed that the non-inflamed ileal mucosa from patients with Crohn's disease have increased epithelial permeability to ovalbumin protein. The authors suggest that this may lead to an increase of antigen load the lamina propria, which could be an initiating pathogenic event in Crohn's disease [Soderholm *et al.* 1999]. More recently, Buhner *et al.* demonstrated that in first degree relatives of Crohn's disease patients, there was a significant association between high mucosal permeability and the presence of a common Crohn's disease associated mutation in CARD15, 3020insC [Buhner *et al.* 2006]. One recent study has implicated dihydroxy bile acids as a negative modulator of intestinal permeability. Normal human colon biopsies mounted in Ussing chambers had increased *E. coli* uptake into the lamina propria in the presence of μM concentrations of dihydroxy bile acids. This study indicats that bile acids could contribute to the development of intestinal inflammation by promoting bacterial uptake in the colon [Munch *et al.* 2007].

1.7 TREATMENT OF CROHN'S DISEASE

1.7.1 Drugs

Drugs of the corticosteroid family (eg prednisolone) are commonly used to treat Crohn's disease due to their anti-inflammatory effects on immunoregulatory T cells [Paliogianni *et al.* 1993], monocytes [Breuninger *et al.* 1993], macrophages [Linden *et al.* 1994] and dendritic cells [de Jong *et al.* 1999]. Drugs such as methotrexate or azathioprine are sometimes used as a treatment to suppress the immune system. Infliximab is a chiral monoclonal IgG1 antibody to tumour necrosis factor, used in more severe cases of Crohn's disease [Rutgeerts *et al.* 2006; Van Assche *et al.* 2006; Juillerat *et al.* 2007].

1.7.2 Antibiotics

Several chronic granulomatous diseases have been successfully treated with antibiotics, including Whipple's disease, malakoplakia, and granulomatous colitis of Boxer dogs [Van Kruiningen 1995], suggesting that antibiotic treatment might be beneficial for Crohn's disease. Several independent trials have investigated this hypothesis, and revealed interesting findings.

In active Crohn's disease, metronidazole has been shown to be a useful treatment for patients with colonic Crohn's disease; 6/22 patients investigated in randomized double-blind cross-over trial showed significant improvement following antibiotic treatment [Blichfeldt *et al.* 1978].

Ciprofloxacin, in a randomised trial against active Crohn's disease, was found to be as effective as a treatment as mesalazine for treating mild to moderate relapses of Crohn's disease [Colombel *et al.* 1999], whilst a study by Leiper *et*

al. demonstrated that clarithromycin treatment for 3 months was ineffective in active Crohn's disease, but that a possible benefit was detected at 1 month, with the suggestion being that the initial beneficial effect of the antibiotic could be attenuated by subsequent bacterial resistance [Leiper *et al.* 2008].

Treatment with both metronidazole and ciprofloxacin led to clinical remission following a 12-week treatment period in 45.5% of patients investigated, compared with 63% for those patients receiving steroid treatment [Prantera *et al.* 1996]; however, a retrospective study of this combination of antibiotics found no benefit compared to single antibiotic therapy; 70.6% of patients taking the two antibiotics achieved clinical remission, 72.8% with metronidazole alone, and 69.0% with ciprofloxacin alone [Prantera *et al.* 1998]. A meta-analysis, conducted by Rahimi *et al.*, examined 6 randomized, placebocontrolled trials conducted with metronidazole, ciprofloxacin and cotrimoxazole – they concluded that patients receiving antibacterial therapy were 2.257 times more likely to have clinical improvement compared with those receiving placebo [Rahimi *et al.* 2006].

Ornidazole, in an open trial, led to 75% clinical remission following 4 weeks of treatment [Triantafillidis *et al.* 1996], and treatment with rifaximin for 16 weeks also decreased disease activity [Guslandi 2005].

Treatment with the antimycobacterials rifampicin and / or ethambutol did not lead to clinical remission [Shaffer *et al.* 1984], nor did Rifampicin and / or Isoniazid and / or ethambutol treatment [Swift *et al.* 1994; Thomas *et al.* 1998].

1.7.3 Probiotics

Probiotics are viable microorganisms that confer therapeutic benefit beyond their nutritional value. Data from human studies suggest the presence of intestinal dysbiosis in Crohn's disease, and therefore the use of probiotic could provide a rational approach to redress this balance, and perhaps aid treatment. Clinical trials conducted so far using probiotic microorganisms have revealed some interesting results.

Trials using *Saccharomyces boulardii* as a probiotic agent led to a significant reduction in the frequency of bowel movements in Crohn's disease patients [Plein *et al.* 1993] in a randomized double-blind placebo-controlled trial, whilst the rate of relapse or in Crohn's disease was significantly reduced when *Saccharomyces boulardii* was used in combination with mesalamine as a treatment strategy to maintain remission [Guslandi 2000].

Lactobacillus GG led to a significant improvement of clinical activity and intestinal permeability in children with mildly active Crohn's disease [Gupta *et al.* 2000].

E. coli strain Nissle 1917 has been tested as a potential probiotic for use in Crohn's disease in several randomized double-blind trials. One such trial measured maintenance of remission in Crohn's disease following surgery, where is was observed that, whilst there was no difference in the number of patients obtaining remission from symptoms, there were fewer relapses for patients on probiotic medication, implying a potential use in maintenance therapy [Malchow 1997].

1.8 SUMMARY

There is convincing evidence supporting the concept that Crohn's disease is a multi-factorial disease and that genetically susceptible hosts have aberrant immune responses and loss of tolerance to environmental factors which contribute to the mucosal inflammation. Functional defects in the epithelial barrier have been suggested which allow inappropriate or unusual access of both bacterial and other microbial antigens to the lamina propria, as a possible consequence of inflammation, and also allowing further propagation of inflammation already seen. In the healthy homeostatic gut, commensal bacteria encountering the lamina propria would typically mediate a poor inflammatory response due to induction of host tolerance. In Crohn's disease, it is plausible that defects in innate and/or adaptive immune cells somehow lose this tolerance, and give rise to a non self-limiting chronic inflammation as a result of bacterial / other antigens encountering the lamina propria. It is possible that AIEC may play an important role in any of these processes.

1.9 HYPOTHESIS FOR THE ROLE OF AIEC IN CROHN'S DISEASE PATHOGENESIS

1. Crohn's disease AIEC may play a role in Crohn's disease pathogenesis by adhering to and invading into intestinal epithelial cells, from where an aberrant immune response can be initiated.

2. Since the initial lesions of Crohn's disease frequently occur in the region of M-cells, typically over lymphoid aggregates in the large bowel, and over Peyer's patches in the small bowel, AIEC may be translocated at these points to the underlying lamina propria, leading to formation of aphthous lesions. This process may be exacerbated by dietary components which affect intestinal barrier function.

3. The processes of AIEC adhesion, invasion and translocation to the gut may be prevented by dietary components, such as dietary fibre. This may have considerable therapeutic potential.

4. Crohn's disease may occur as a result of defective clearance of ingested gut microbiota by phagocytes. AIEC may survive and replicate with macrophages resulting in the granulomatous lesions of Crohn's disease. Antibiotics may be used to inhibit these processes.

1.10 AIMS

1. To assess AIEC adhesion and invasion to colonic epithelial cell lines *in vitro.*

2. To determine if AIEC can be translocated across intestinal M-cells *in vitro.*

3. To assess the role of dietary components such as dietary non-starch polysaccharides (NSP) and food emulsifiers on AIEC translocation across M-cells.

4. To assess the ability of AIEC to survive within macrophages

5. To assess the efficacy of combination antibiotic treatment against internalised AIEC within macrophages.

Chapter 2

Methods

2.1 MATERIALS

Unless otherwise stated, all chemicals used herein were purchased from Sigma-Aldrich (Sigma-Aldrich Company Ltd, Gillingham, UK). Bacto™ agar, Bacto™ tryptone and Bacto™ yeast extract for use in bacterial culture were obtained from BD Biosciences (Becton, Dickinson and Company, Oxford, UK). Transwell® filters used in M-cell generation were obtained from Millipore (Millipore (UK) Limited, Watford, UK). Osmium tetroxide, glutaraldehyde, and araldite resin, all used in sample preparation for electron microscopy, were obtained from Agar Scientific (Stansted, United Kingdom).

All plastics used for human cell culture were obtained from Corning® (Corning Incorporated, Corning, USA) as were plastics used in liquid bacterial culture. Solid bacterial culture was performed using plastics obtained from Sterilin (Sterilin Limited, Caerphilly, UK).

Soluble non-starch polysaccharides (plantain, broccoli, leek, and apple) used in bacterial cell adhesion and translocation assays were provided by Provexis plc (Liverpool, UK). The food emulsifiers (polysorbate-60 and polysorbate-80) were obtained from Sigma-Aldrich (Sigma-Aldrich Company Ltd, Gillingham, UK).

The antibiotic clarithromycin was obtained from Abbott (Abbott Laboratories Ltd., Queenborough, United Kingdom), all other antibiotics were obtained from Sigma-Aldrich (Sigma-Aldrich Company Ltd, Gillingham, UK).

2.2 BACTERIAL STRAINS AND GROWTH CONDITIONS

2.2.1 Crohn's disease and control patient population *E. coli* isolation

E. coli were previously isolated from patients with Crohn's disease or from a control population comprising of patients with irritable bowel syndrome (IBS) and sporadic polyps, as described elsewhere [Martin 2004; Martin *et al.* 2004]. Briefly, colonic biopsy specimens were washed in PBS and treated with 500 µL 0.016% dithiotreitol (DTT) solution for 15 minutes to remove the overlying mucus layer; 50 µL of the resultant DTT solution was plated onto MacConkey agar and incubated at 37°C for 24 hours – bacterial growth on this plate was assumed to that of mucus-associated bacteria. Following dithiothreitol treatment, the biopsy samples were transferred into 1.5 mL Eppendorf tubes containing either 500 µL sterile saline or 500 µL gentamicin (50 µg / mL) to kill any extracellular bacteria, for 30 minutes. The biopsies were then serially washed in PBS. The gentamicin treated biopsy was then lysed in sterile distilled and de-ionised water, and again, the supernatant plated onto MacConkey agar and incubated at 37°C for 24 hours – bacteria on this plate were assumed to be intracellular bacteria. The supernatant from the PBS treated biopsy was then plated onto MacConkey agar and incubated at 37°C for 24 hours, bacteria on these plates were assumed to be adherent to the biopsy cell surface.

Following the 24 hour incubation, the bacterial colonies were characterised morphologically by Gram staining, and Gram-negative rods were further characterised by using API20E bacterial identification kits (Bio-Merieux, Marcy L'Etolie, France). In all, thirteen of these *E. coli* isolates, representing six from the control group, and seven from the Crohn's disease group, were selected for further study in this work; their known characteristics are summarised in Appendix 1.

2.2.2 Bacteria from other sources

In addition to the colonic *E. coli* isolates previously identified in our lab, several other bacterial strains were utilised from other sources as controls in some experimental work. These included the ileal Crohn's disease mucosa-associated *E. coli* isolate LF82, kindly donated by Professor A. Darfeuille-Michaud (Pathogenie Bacterienne Intestinale, Laboratorie de Bacteriologie, Faculte de Pharmacie, Clermont-Ferrand, France). LF82 had previously been described as an adherent and invasive *E. coli* (AIEC) as it was found to adhere to and invade into Intestine-407 (I407) epithelial cells lines *in vitro*, and was found to replicate within both epithelial cells and macrophages [Boudeau *et al.* 1999; Darfeuille-Michaud 2002]. In addition, *E. coli* K12, obtained from the American Type Culture Collection (ATCC®) (LGC Standards, Middlesex, UK; ATCC® Number 10798 [Tompkins *et al.* 1997]) was used as a control in bacterial translocation across M-cell and control Caco2-cl1 monolayers to indicate low level rates of translocation, as was the probiotic *E. coli* Nissle 1917, a kind gift from Dr C Enders (ArdeyPharm, Herdecke, Germany). *Salmonella* Typhimurium LT2 and *Shigella sonnei* were a kind gift from Dr C. Winstanley (School and Infection and Host Defence, Faculty of Medicine, University of Liverpool, England) and were used as positive controls in M-cell translocation assays.

2.2.3 Bacterial growth conditions

2.2.3.1 Solid agar culture

For most assays, bacteria were simply grown from frozen stocks onto solid Luria-Bertani (LB) agar plates (Appendix 2) at 37°C, for 24 hours. Following this, a group of approximately 5 colonies were removed from the plate, suspended in sterile saline, centrifuged at 10,000 x *g* for 10 minutes, and washed three times in sterile saline. *E. coli* were then suspended to an OD at

550nm of 0.825, equivalent to 1 x 10^6 *E. coli* per µL, and used immediately as the assay required.

2.2.3.2 Broth-based culture

Bacteria were grown as for solid agar culture; following the 24 hour incubation at 37°C, a single colony was removed and suspended in 10 mL of LB broth (Appendix 2). This was then incubated at 37°C in an orbital incubator (Sanyo, Gallenkemp PLC, Loughborough, UK) at 200 rpm overnight. Following this, 10 mL of fresh LB broth was then inoculated with 100 µL of the bacterial suspension, and incubated in the orbital incubator at 37°C for approximately 2 hours until Log phase growth was achieved. The bacterial suspension was then centrifuged at 10,000 x *g* for 10 minutes, and washed three times in sterile saline. *E. coli* were then suspended to an OD at 550nm of 0.825, equivalent to 1 x 10^6 *E. coli* per µL (based on McFarland standards, Pro-Lab Diagnostics, Cheshire, UK), and used immediately as the assay required.

2.2.3.3 Sterile PBS culture

Bacteria were grown as for solid agar culture; following the 24 hour incubation at 37°C, a single colony was removed and suspended in 100µL sterile PBS, and left at room temperature for 72 – 96 hours. The bacterial suspension was then centrifuged at 10,000 x *g* for 10 minutes, and washed three times in sterile saline. *E. coli* were then suspended to an OD at 550nm of 0.825, equivalent to 1 x 10^6 *E. coli* per µL, and used immediately as the assay required.

2.3 CELL CULTURE: CELL LINE MAINTANENCE

2.3.1 Culture of Caco2 cells

The adherent human colonic epithelial cell line Caco2 (ATCC® Number HTB-37, [Trainer *et al.* 1988]) was maintained at 20% – 80% confluency in DMEM supplemented with 10% FCS, 100 U / mL penicillin, 100 µg / mL streptomycin and 8 mM glutamine (complete DMEM). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2, 95% air. Used at sub confluent levels, these cells are models of isolated colonic epithelial cells. Upon reaching confluencey, the cells express characteristics of enterocytic differentiation, and thus are models of the colonic epithelial barrier. These cells are heterogenous for microvillar length and microvillar aggregation

2.3.2 Culture of Caco2-cl1 cells

The adherent human colonic epithelial cell line Caco2-cl1 was a kind gift from Dr E. Gullberg (Colorectal Surgery Unit, Department of Surgery, University Hospital, SE-581 85 Linköping, Sweden [Gullberg *et al.* 2000]). These cells were maintained at 20% – 80% confluency in DMEM supplemented with 10% FCS, 100 U / mL penicillin, 100 µg / mL streptomycin and 8 mM glutamine (complete DMEM). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2, 95% air.

Like the Caco2 parent cell line, upon reaching confluencey, these Caco2-cl1 cells express characteristics of enterocytic differentiation, and thus are models of the colonic epithelial barrier. In addition, this cell line has exclusive apical villin localisation. Twenty-one days post confluency, Caco2-cl1 cells form a polarized monolayer with an apical brush border (BB) morphologically comparable to that of the human colon. The isolated BB contains the

microvillar proteins villin, fimbrin, sucrase-isomaltase, myosin-1, fodrin and myosin II, at levels similar to that of the human enterocyte. These cells are still heterogenous for microvillar length and microvillar aggregation

2.3.3 Culture of Raji B cells

Raji B cells were a kind gift from Mrs C. Thomas (School of Clinical Sciences, University of Liverpool, Liverpool, UK. L69 3BX; ATCC® Number CCL-86, [Pulvertaft 1964]). These non-adherent cells are a cell line model of B lymphocytes, and were used to facilitate transformation of Caco2-cl1 cells into cells with an M-cell phenotype. The Raji B cells were maintained at 10 – 30% confluency in Roswell Park Memorial Institute-1640 media (RPMI-1640) supplemented with 10% FCS, 100 U / mL penicillin, 100 µg / mL streptomycin and 4 mM glutamine (complete RPMI-1640). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2, 95% air.

2.3.4 Culture of J774-1A cells

The murine macrophage-like cell J774-A1 was obtained from the ATCC (ATCC® Number TIB-67 [Ralph *et al.* 1975]). Cells were maintained at 25 – 50% confluency, in RPMI-1640 media supplemented with 10% foetal calf serum (FCS), 100 U / mL penicillin, 100 µg / mL streptomycin and 4 mM glutamine (complete RPMI-1640). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2, 95% air.

2.4 BACTERIAL ADHERENCE AND INVASION OF INTESTINAL EPITHELIAL CELLS

2.4.1 Cell lines

Three intestinal cell line models were used to establish bacterial adhesion and invasion into intestinal epithelial cells: I407, Caco2, and Caco2-cl1. I407 were selected as they had previously been used by Darfeuille-Michaud and colleagues as a reference epithelial cell line for AIEC adherence and invasion [Boudeau *et al.* 1999] and by Martin and colleagues [Martin *et al.* 2004]. Caco2 and Caco2-cl1 cells were selected as a model of colonic epithelial cells.

2.4.2 Plant fibres

Soluble plant non-starch polysaccharides (NSP) have previously been investigated for their ability to block the attachment of adherent and invasive *E. coli* (AIEC) to intestinal epithelial cells *in vitro* (I407 and HT-29 cells) [Martin *et al.* 2004]. Soluble NSP from plantains were found to be particularly potent (plantains are a banana family (*Musa*) member that are usually cooked as a vegetable). Soluble plant fibres contain a wide range of oligosaccharide structures and might have the ability to inhibit a wide range of bacteria-epithelial interactions. The plant NSPs tested here, namely apple, plantain [*Musa* spp i.e. the banana family], broccoli and leek were provided by Provexis PLC. The plant NSP were dissolved in DMEM (without FCS, penicillin, streptomycin and glutamine) at a concentration of 500 mg / mL, followed by sterilisation through a 0.2 µm filter, and then used at a final concentration of 0 – 50 mg / mL.

2.4.3 *In vitro* invasion assays

Seven colonic Crohn's disease AIEC isolates, six control patient AIEC, the ileal AIEC isolate LF82, and *E. coli* K12 (Appendix 1) were tested for their ability to invade the intestinal cell line I407, and the colonic cell lines Caco2 and Caco2-cl1 cells. *Shigella sonnei* was also used with the intention that it would act as a positive control for invasion.

Cells were maintained as described earlier. After cells had reached confluency levels of approximately 60%, they were detached from their flasks by treatment with 1 x trypsin-EDTA solution (0.05% trypsin (v/v), 0.02% EDTA (w/v) in PBS) and seeded into 24-well tissue culture treated plates (Costar, High Wycombe, UK), at a density of 1 x 10^5 cells per well in 1 mL; cells were maintained in complete DMEM for 24 hours. The cells were then washed twice with sterile PBS prewarmed to 37°C, and the media replaced with fresh complete DMEM but lacking antibiotics. *E. coli,* prepared as per 'solid agar culture', were added at a concentration of 1 x 10^6 *E. coli* per well (1:10 dilution of an OD 0.825 at 550nm solution, 10 µL of the resultant solution were added per well). *E. coli* were incubated with cells in wells for 4 hours at 37°C, 5% CO_2 in a humidified atmosphere. Wells were then washed three times in sterile PBS. To determine bacterial invasion, wells were treated with fresh culture medium containing gentamicin at 100 µg / mL to kill any extracellular bacteria. After one hour at 37°C, the wells were washed three times in sterile PBS. Cells were lysed by adding deionised water containing 1% Triton-X100 (v/v) for 5 minutes to release internalised bacteria. Eight 10-fold dilutions of the cell lysate were performed, and 50µL from each was plated onto LB agar plates in triplicate. Plates were incubated at 37°C for 24 hours, and colony forming units (CFU) counted. One colony forming unit originates from a single bacterium which has proliferated to become a visible colony.

2.4.4 *In vitro* adhesion assays

Three colonic Crohn's disease AIEC isolates, three control patient AIEC, the ileal AIEC isolate LF82, and *E. coli* K12 (Appendix 1) were tested for their ability to adhere to the intestinal cell line I407. The method to determine bacterial adhesion follows the same protocol as determining bacterial invasion, with the omission of the gentamicin step, with bacteria and cells incubated together for a total of 4 hours.

2.4.5 Adhesion and invasion assays in the presence of soluble non-starch polysaccharides (NSPs)

Several strains of *E. coli* were subjected to further investigation to determine if the presence of soluble non-starch polysaccharides (NSP) such as plantain or broccoli could prevent or disrupt bacterial adhesion and invasion into Caco2-cl1 cells. The protocol was the same as for the standard invasion and adhesion assay described previously, with the exception that the cell media were supplemented with NSPs over a range of concentrations from 0 mg / mL – 50 mg / mL (w/v), and was added to the cells 30 minutes prior to the addition of the bacteria.

2.5 M-CELL AND Caco2-CL1 MONOLAYER GENERATION

2.5.1 Transwell® filter set up

The apical surface of 12mm (0.6 cm^2) Transwell® filters (Millicell® Culture Plate Inserts with 3.0μm pores (Millicell, cat. No. PITP 012 50)) were coated with 13.56 μg Matrigel™ (Becton, Dickinson and Company, Oxford, UK) diluted in 300 μL ice-cold DMEM (without FCS, penicillin, streptomycin and glutamine). Transwells® were then left for 1 hour at room temperature in sterile conditions to allow the Matrigel™ to gel. Transwells® were then washed twice with DMEM.

2.5.2 Caco2-cl1 cell seeding

Caco2-cl1 cells were seeded into the apical Transwell® chamber at 5 x 10^5 cells in 500 μL complete DMEM. Wells were placed in 12 well plates and 1500 μL complete DMEM added to the basolateral aspect. Caco2-cl1 cells were left to adhere overnight, followed by replacement of all media with complete DMEM; subsequently, both apical and basolateral media was changed every other day. Caco2-cl1 cells were cultured for 14 - 16 days at 37°C in a humidified atmosphere of 5% CO_2, 95% air, until confluent monolayers were formed,

2.5.3 Detection of confluent monolayers

2.5.3.1 Transepithelial Electrical Resistance (TEER)

Confluent Caco2-cl1 monolayers were monitored daily by measuring transepithelial electrical resistance (TEER) using an epithelial voltohmmeter (World Precision Instruments, Hertfordshire, UK). Cultures which had TEER, values < 300 Ωcm^2 at day 16 of culture were discarded and not used in any assay.

2.5.3.2 Light Microscopy

Twelve well plates with a growth area of 3.8 cm^2 / well were coated with Matrigel at the same concentration as Transwell® filters (85.88 µg Matrigel™ in 1.9 mL ice cold DMEM per 12-well plate well) and left to gel for 1 hour at room temperature. Caco2-cl1 cells were seeded at a concentration of 1 x 10^6 cells / mL, in a volume of 3.17 mL. The growth of the monolayers was monotored via light microscopy. This allowed direct visualisation of Caco2-cl1 cell growth on Matrigel™, since Caco2-cl1 cells growing on the Transwell® filter could not be easily monitored due to the filter itself preventing clear visualisation of the cells.

2.5.4 Caco2-cl1 monolayer formation

Upon reaching TEER, values > 300 Ωcm^2 at day 14 – 16 of culture, control Transwells® were then cultured for a further 4 – 6 days, with the apical and basolateral media changed each day. TEER was monitored throughout – any cultures with TEER < 300 Ωcm^2 were discarded and not utilised for further study.

2.5.5 M-cell monolayer generation

Raji B cells were removed from complete RPMI-1640 medium, centrifuged at 1,000 x *g* for 5 minutes and resuspended in complete DMEM to a final concentration of 333,000 cells / mL; 1.5 mL of this medium was then added to the basolateral chamber of the Transwell®. Raji B – Caco2-cl1 cells were cocultures together for a further 4 - 6 days. Apical and basolateral media was changed each day.

2.5.5 Monolayer preparation for assays

Prior to use, Caco2-cl1 and M-cell monolayers were removed from complete DMEM and Raji B cells, washed once in DMEM, and twice in sterile PBS at 37°C, media was them replaced with DMEM without penicillin and streptomycin, and left for 1 hour before use.

2.6 BACTERIAL TRANSLOCATION ACROSS Caco-2 AND M-CELL MONOLAYERS

2.6.1 Translocation of bacterial species

The apical surface of Caco2-cl1 and M-cell monolayers was infected with 10µL bacterial suspension containing 1 x 10^7 bacterial colony forming units (CFU). Aliquots from the basolateral media were removed at appropriate time points (0 – 24 hours), and the bacterial load quantified; ten-fold dilutions of the basolateral media were performed, and 50 µL from each was cultured on LB agar plates, incubated at 37°C, 5% CO_2 and colony forming units were counted after 24 h.

Following removal of the required aliquots of basolateral media, all basolateral media was removed from surrounding the well, and from below

the monolayer itself. The filter grown monolayers were then incubated for a further hour at 37°C, any Transwells® which had basolateral media present at this point were discard, as it was assumed that the basolateral media had leaked from the apical chamber – as such, translocation data for these wells would be unreliable, and was discarded. In addition, TEER was monitored throughout, those wells with TEER < 300 Ωcm^2 at any point were discounted as this indicted disruption to the monolayer integrity.

Within each translocation assay, *E. coli* K12 (having a low translocation rate) and Crohn's disease AIEC HM605 (having a high translocation rate) were always included to assess interassay variability. Data was presented as the total number of bacterial species translocating across monolayers per cm^2 of monolayer.

2.6.2 Preparation of dietary components.

Plant NSPs were prepared and used as described previously (section 2.4.2). The food emulsifiers polysorbate-80 and polysorbate-60 were diluted in DMEM (without FCS, penicillin, streptomycin and glutamine) at 37°C to ensure solubilisation at a concentration of 1% polysorbate-60 (v/v) and 0.1% polysorbate-80 (v/v). The polysorbates were then used at a final concentration of 0.1% - 0.0001% polysorbate-60 and 0.01% - 0.0001% (v/v) polysorbate-80 (the range predicted to be physiologically relevant).

2.6.3 Translocation in the presence of dietary components.

To assess the impact of dietary factors such as fibres and emulsifiers upon translocation, prior to infection with bacterial species, the apical surface of the monolayers were pre-incubated for 30 minutes with fibre or emulsifiers

prior to infection. After this 30 minute pre-incubation period, monolayers were infected in the normal way, and bacterial translocation quantified as usual.

2.7 TRANSMISSION ELECTRON MICROSCOPY OF Caco2-CL1 AND M-CELL MONOLAYERS

2.7.1 Sample preparation and embedding in resin

Transwells® containing Caco2-cl1 cells or M-cell monolayers before or during infection were prepared for transmission electron microscopy. Transwells® were washed 2 x 5minute PBS, and then fixed in 2% glutaraldehyde (w/v) and 4% paraformaldehyde (w/v) in PBS for 1 h at RT. This was followed by 2 x 15 minute PBS washes, then 1 hour at room temperature in 1% osmium tetroxide (v/v), and then 2 x 30 minute PBS and 1 overnight PBS wash. The following morning, the PBS was removed from the monolayers by 2 x 15 minute sterile H_20 washes. Monolayers were then serially dehydrated by a series of ethanol washes; 20 minutes in 30% ethanol, then 10 minutes each in 60%, 70%, 80%, and 90% ethanol, finally followed by 2 x 15 minute washes in 100% ethanol. The cell monolayer coated filter was then carefully removed from the Transwell®, using a scalpel, and cut into 3 mm wide strips (ensuring the cell monolayer is uppermost). Samples were then incubated for 30 minutes in 50% ethanol, 50% araldite resin, and then for 1 hour in araldite resin. The samples were then transferred to moulds containing 100% resin, and baked in a dry oven at 60°C for 48 hours.

2.7.2 Sample sectioning

Once polymerised, blocks were trimmed at the sample containing end to an area of 0.25mm^2. Samples were then sectioned on a Reichert Ultracut E (Reichert Analytical Instruments, Depew, USA) using a diamond knife (DiATOME AG, Biel, Switzerland) to a thickness of 70nm. Samples were then mounted onto copper grids, and left to dry for at least 24 hours before staining.

2.7.3 Sample staining

Copper grids were incubated, sample side down, on 100µL drops of Reynards Lead Citrate (Appendix 2) for 5 minutes, followed by 3 x 20 second vigorous washes in distilled water. Samples were then incubated, again, sample side down, on 100µL drops of 5% uranyl acetate (w/v) for 5 minutes, followed by 3 x 20 second vigorous washes in distilled water. Samples were left to air dry for at least 24 hours.

2.7.4 TEM analysis

Samples were examined using a FEI 120kV Tecnai G 2 Spirit BioTWIN transmission electron microscope (Biomedical Electron Microscopy Unit, University of Liverpool, Liverpool, UK).

2.8 MONOLAYER EXAMINATION BY IMMUNOBLOTTING

2.8.1 Sample preparation

Caco2-cl1 and M-cell monolayers were washed three time in sterile PBS, the PBS was removed and the monolayers immediately frozen at -80 °C overnight

to lyse the cells. The cell lysates were then resuspended in 250 µL sample buffer, vortexed for 30 seconds to ensure a homogeneous sample and then boiled at 90 °C for 5 minutes.

2.8.2 Immunoblotting

Solubilised proteins were separated by SDS-PAGE on 10% resolving, 4% stacking polyacrylamide gel (Appendix 2) and electrotransferred to nitrocellulaose membrane for 1 hour, at 100 V, in 25 mM Tris, 192 mM glycine and 20% (v/v) methanol.

2.8.3 *Aleuria aurantia* lectin binding

Aleuria aurantia lectin (Catalog number B-1396; Vector laboratories, Peterborough, UK) has been found to target human M-cells [Roth-Walter *et al.* 2004; Roth-Walter *et al.* 2005]. It has been used here to detect M-cell generation from Caco2-cl1 cells.

Following M-cell and Caco2-cl1 protein transfer onto nitrocellulaose membrane, the membrane was washed in 50 mM Tris, pH 7.5, 0.15M NaCl, 0.1% Tween-20 (v/v), blocked in buffer containing 1% BSA (w/v), incubated with a biotinylated *Aleuria aurantia* lectin diluted 1:2000 in buffer, washed in buffer, and detected using peroxidase-conjugated avidin (Bio-Rad, Hemel Hempstead, UK), and this detected using a chemiluminescence Super-signal immunoblotting detection kit (Pierce, Rockford IL, USA).

2.8.4 Alkaline Phosphatase protein expression

Alkaline Phosphatase expression is known to be down regulated in M-cell cultures compared with Caco2 / Caco2-cl1 cultures [Gullberg *et al.* 2000; Tyrer *et al.* 2002; Lai *et al.* 2008]. For this reason, alkaline phosphatase expression was assessed as an indicator of M-cell generation from Caco2-cl1 cells.

Following M-cell and Caco2-cl1 protein transfer onto nitrocellulose membrane, the membrane was blocked with Tris-buffered saline (TBS) containing 1% (w/v) BSA, 0.1 % (v/v) Tween 20 for 1 hour. The membrane was then probed with the primary antibody; rabbit polyclonal to human intestinal alkaline phosphatase ([ab7322], Abcam; Cambridge, UK) diluted 1:5000 in TBS – 0.1 % Tween 20 (v/v) (TBS Tween) for 1 hour, followed by 3 x 20 minute washes in TBS Tween. Peroxidase-conjugated secondary anti-rabbit Ig antibody at 1:5000 was used (Dako; Glostrup, Denmark). Alkaline phosphatase expression was detected using the Super-signal immunoblotting detection kit (Pierce; Rockford IL, USA).

2.8.5 Pan-actin expression to detect equal loading of protein samples

Equal loading of samples for western blot analysis was confirmed using a pan-actin antibody (1:500) (Antibody #4968; Cell Signalling Technology®; Danvers, MA, USA). Membranes were washed three time in TBS tween, incubated with the primary antibody for 1 hour, washed in TBS tween, followed by appropriate secondary antibody incubation for 1 hour, and detected again using the Super-signal immunoblotting detection kit.

2.8.6 Antibody / Lectin detection

Following use of the Super-signal immunoblotting detection kit, membranes were examined using a Bio-Rad Fluor-S MultiImager (Bio-Rad Laboratories, California, USA), a quantitative imaging system for capturing high-resolution digital images. Using Quantity One® software (Bio-Rad Laboratories, California, USA), images were subjected to quantitative densitometric analysis of protein bands.

2.9 BACTERIAL INTERACTIONS WITH J774-A1 MACROPHAGE CELLS

2.9.1 Bacterial replication within J774-A1 macrophage cells

Murine macrophage cells J774-A1 were seeded into 24-well tissue culture plates at density of 1 x 10^5 cells per well in 1 mL and maintained in complete media (RPMI-1640) for 24 hours. The wells were then washed three times in sterile PBS. *E. coli* were added at a concentration of 1 x 10^6 *E. coli* per well (1:10 dilution of a OD 0.825 at 550nm solution, 10µL of the resultant solution were added per well). *E. coli* were incubated with cells in wells for 2 hours at 37°C, 5% CO_2 in a humidified atmosphere. Following this, wells were washed three times in sterile PBS to remove non-adherent bacteria. Wells were then treated with fresh culture medium containing gentamicin at 20 µg / mL for a further hour, to both kill any extracellular bacteria [Subramanian *et al.* 2008]. Following this, the media was removed and replaced with complete media lacking antibiotics, and left for a further 3h to allow replication of internalised bacteria. Subsequently, the wells were washed three times with sterile PBS, and the cells lysed by adding deionised water containgin 1% v/v Triton-X100 (v/v) for 5 minutes to release internalised bacteria. Ten-fold dilutions of the cell lysate were performed, and 50µL from each was plated onto LB agar plates. Plates were incubated at 37°C for 24 hours, and colony forming units

(CFU) counted, with a CFU taken to represent a single viable *E. coli* from within the macrophage cell. To calculate the percentage of replication, the viable CFU counts at the end of the three hour replication period were compared to viable CFUs obtained immediately after one hour of gentamicin treatment.

2.9.2 Efficacy of antibiotics against bacteria internalised within macrophage cells

To assess the efficacy of antibiotics against internalised bacteria, a representative Crohn's disease associated AIEC was selected. This *E. coli,* HM605, was then used for further study as it had previously been shown to replicate extensively within macrophages. Cells were infected and treated with gentamicin as described above. Following gentamicin treatment, cells were incubated with various antibiotics, such as rafampicin, clarithromycin, tetracycline, trimethoprim or ciprofloxacin, both as single treatments, and in combination with each other, for 3h. Antibiotics were tested at commonly observed peak serum concentrations (C_{max}), and at 10% of C_{max}. Cells were lysed and plated as described above to yield CFU and % killing of bacteria was determined as compared to control level survival.

2.9.3 Transmission electron microscopy (TEM) of macrophages

2.9.3.1 Sample preparation and embedding in resin

Subsequent to infection, as described above, J774-A1 cells were fixed in 2% glutaraldehyde and 4% paraformaldehyde (both Agar Scientific) in PBS, at room temperature (RT) for 1 hour. Cells were then washed in PBS for 5minutes, and left overnight at 4°C in fresh PBS. The following morning, cells were again washed in PBS, followed by 2 x 15 minute washes in 0.15M

glycine in PBS, followed by a 5 minute PBS wash. Cells were then gently scraped into an Eppendorf containing 1 mL PBS. Cells were then centrifuged at 5000 x g for 2 minutes. The resultant pellet was then suspended in 2% liquid agarose, centrifuged at 5000 x g for 2 minutes, and left upon ice until set. Samples were then cut into $2mm^3$ pieces. Samples were further fixed in 1% osmium tetroxide, which also gives contrast when viewed under TEM, for a further 1 hour. This was followed by 2 x 30 minute PBS washes, 1 x 15 minute distilled water wash, then 1 x 30 minute 30% ethanol in water (ethanol wash). The samples were then left for 1 hour in 30% ethanol containing an additional 0.5% urayl acetate. Following this step, the samples were washed for 10 minutes each in increasing strength ethanol washes (30%, 60%, 70%, 80%, 90%) followed by 2 x 15 minute washes in 100% ethanol. Samples were then washed in 1:1 acetone:ethanol mix for 10 minutes, followed by a 30 minute 1:1 acetone:resin mix, and finally a 100% resin was added. Samples were placed in moulds 3mm x 4mm x 9mm along with fresh resin, and then polymerised in an oven at 60°C for 24 – 48 hours.

2.9.3.2 Sample sectioning

Once polymerised, blocks were trimmed at the sample containing end to an area of $0.25mm^2$. Samples were then sectioned as per section 2.7.2, stained as per section 2.7.3 and examined as per section 2.7.4.

2.9.4 Effect of antibiotics on macrophage viability

Murine macrophage cells J774-A1 were seeded into 24-well tissue culture plates at density of 1×10^5 cells per well in 1 mL and maintained in complete RPMI-1640 for 24 hours. The wells were then washed three times in sterile PBS. Antibiotics, at C_{max} of each antibiotic, were then added to each well and incubated at 37°C, 5% CO_2 in a humidified atmosphere for 3h. Cells were then

detached from their well by treatment with 1 x trypsin-EDTA solution (0.05% trypsin (v/v), 0.02% EDTA (w/v) in PBS) and re-suspended in 0.9 mL PBS, and Trypan-blue added to a final concentration of 1 mg / mL (w / v). Macrophages were then counted using a hemocytometer, viable cells possess intact cell membranes that exclude Trypan-blue, appearing colourless, whereas dead cells do not, appearing blue under microscopic examination.

2.10 STATISTICAL ANALYSIS

All statistical analysis was performed using StatsDirect software (StatsDirect v2.3.1; Sale, UK). Independent sample groups were assessed for normality using the Shapiro-Wilk W test [Shapiro *et al.* 1965]. Samples with a normal distribution were tested for equality of variance using Levene's test [Brown 1974]. Data sets consisting of multiple treatments which were found to be both normally distributed and of equal variance were analysed using the parametric one-way analysis of variance (ANOVA), followed by pair-wise comparisons of treatment means; data sets consisting of 2 samples were analysed using Student's t-test. Multiple treatment data sets found to be either non-normally distributed, or displaying unequal variances were analysed using non-parametric one-way (Kruskal-Wallis) ANOVA [Critchlow 1991], significance of data was determined using Conover tables [Conover 1999]; Mann-Whitney U was used when data consisted of only 2 samples. In the ANOVA, Student's t-test, Kruskal-Wallis and Mann-Whitney U test, differences were considered significant when $P < 0.05$. All data presented within this thesis is expressed as mean ± standard error of the mean (SEM), unless otherwise stated.

Chapter 3

Adherence and Invasion of Crohn's Disease AIEC to Caco2 and Caco2-cl1 cells, and Inhibition by Plantain NSP

3.1 HYPOTHESIS

Crohn's disease and control patient AIEC isolates are able to invade Caco2 and Caco2-cl1 cells; the AIEC also adhere to these cells. This invasion and adhesion can be inhibited by the presence of soluble plantain fibre.

3.2 AIMS

1. Assess adhesion to and invasion into Caco2 and Caco2-cl1 cells by a panel of Crohn's disease and control patient AIEC.
2. Determine if soluble plantain fibre can prevent this invasion and adhesion.

3.3 INTRODUCTION

It is well accepted due to work by several independent groups that there are increased numbers of mucosa-associated bacteria, particularly *E. coli*, in tissue samples taken from the colonic mucosa of Crohn's disease patients [Swidsinski *et al.* 2002; Martin *et al.* 2004] and from the ileal mucosa of Crohn's disease patients, both adult [Darfeuille-Michaud *et al.* 1998] and paediatric [Conte *et al.* 2006]. These *E. coli,* being either intra-mucosal or mucosa-associated, have been shown to have invasive properties in Crohn's disease patients by several independent groups [Darfeuille-Michaud *et al.* 2004; Martin *et al.* 2004; Baumgart *et al.* 2007; Sasaki *et al.* 2007; Eaves-Pyles *et al.* 2008].

One hypothesis is that *in vivo*, these *E. coli,* termed 'adherent-invasive *Escherichia coli*' [Boudeau *et al.* 1999; Darfeuille-Michaud 2002] adhere to and invade epithelial cells, initiating an inflammatory cascade which gives rise to the characteristic inflammation observed in Crohn's disease. Indeed, *in vitro,* these AIEC have been shown to adhere to, and invade into intestinal cell lines [Darfeuille-Michaud 2002; Martin *et al.* 2004]. Cell lines investigated so far include intestine-407 (I407) [Martin *et al.* 2004], HT-29, an in vitro cell line models of epithelial colorectal adenocarcinoma [Martin *et al.* 2004] and differentiated Caco2 cells [Darfeuille-Michaud *et al.* 1998]. Levels of adhesion and invasion of these AIEC to these cell lines has been shown to be both strain and isolate dependent [Darfeuille-Michaud *et al.* 1998; Martin *et al.* 2004]. Published work to date show that invasion of AIEC strains is most predominant in I407 cells, whilst for many strains invasion in HT-29 cells in very low or absent [Martin *et al.* 2004].

The Liverpool *E. coli* isolates (denoted HM---), unlike isolates from other independent groups had not been tested for their ability to invade Caco2 cells, knowing this would allow for greater understanding of the invasive properties of the Crohn's disease *E. coli* isolates. Additionally, since Caco2-cl1 cells, a sub-clone of Caco2 cells, are commonly used to investigate bacterial translocation assays across epithelial layers, and are used in the generation of *in vitro* M-cells, it would be beneficial to know if Crohn's disease *E. coli* isolates are able to adhere or invade into this Caco2-cl1 cell line.

Previous studies have shown that adhesion of Crohn's disease *E. coli* isolates can be inhibited by the soluble plant fibre plantain NSP [Martin *et al.* 2004]. This work was carried out using the I407 cell line; it is important that this be tested further on a true intestinal epithelial cell line as it potentially could be used a prophylactic therapy for Crohn's disease patients.

3.4 METHODS

A panel of *E. coli* were selected (see Appendix 1) consisting of seven Crohn's disease AIEC, six control patient AIEC, ileal Crohn's disease AIEC LF82, and control *E. coli* K12. These strains were selected to encompass both haemagglutinating and non-haemagglutinating strains within the control and disease group. All strains were investigated for their ability to invade into Caco2 and Caco2-cl1 cells. Three Crohn's disease AIEC displaying the ability to invade Caco2-cl1 cells, ileal Crohn's disease AIEC LF82 and control *E. coli* K12 were then selected for further investigation into the effect that soluble plantain fibre has upon *E. coli* adhesion and invasion into Caco2-cl1 cells. Detailed methods for detection of AIEC invasion and adhesion to Caco2 and Caco2-cl1 can be found within Chapter 2, as can details of the assays using plantain fibre.

Briefly, Caco2 or Caco2-cl1 cells in culture were infected with Crohn's disease or control patient AIEC isolates, for 4 hours, to allow bacterial internalisation, followed by extracellular gentamicin treatment to kill extracellular organisms for 1 hour for invasion studies. Parallel studies in the absence of gentamicin were performed to assess for adhesion. Experiments were conducted with and without incubation with soluble plantain fibre, prior to the addition of bacteria. Cells were lysed, followed by enumeration of invasive or adherent AIEC following overnight growth of the lysate on LB agar.

3.5 RESULTS

3.5.1 AIEC invade Caco2 and Caco2-cl1 cells *in vitro*.

Invasion of the control *E. coli* K12 was found not to be significantly different for Caco2 and Caco2-cl1 cells. All of the AIEC isolates investigated were found to invade into Caco2 cells (Fig 3.1). Of the six control patient AIEC tested, five were found to be more invasive than *E. coli* K12 (being 2.79 – 22.53 fold more invasive than *E. coli* K12). Of the seven Crohn's disease AIEC tested, three were found to invade Caco2 cells to a greater extent than *E. coli* K12 (2.74 – 15.92 fold more invasive). Whilst still invasive, the other strains investigated invaded Caco2 cells less than *E. coli* K12. The classic AIEC LF82 was found to invade Caco2 cells more than K12 (LF82, 2.01 ± 0.83 fold).

All of the AIEC strains investigated were found to invade Caco2-cl1 cells to a greater extent than *E. coli* K12 (Fig 3.2) (2.72 – 25.55 fold more invasive). *E. coli* LF82 was also more invasive than *E. coli* K12 (1.58 ± 0.06 fold). The *E. coli* strains appeared to be more invasive in the Caco2-cl1 cell line than the Caco2 cells line, as all strains investigated were more invasive of Caco2-cl1 than *E. coli* K12.

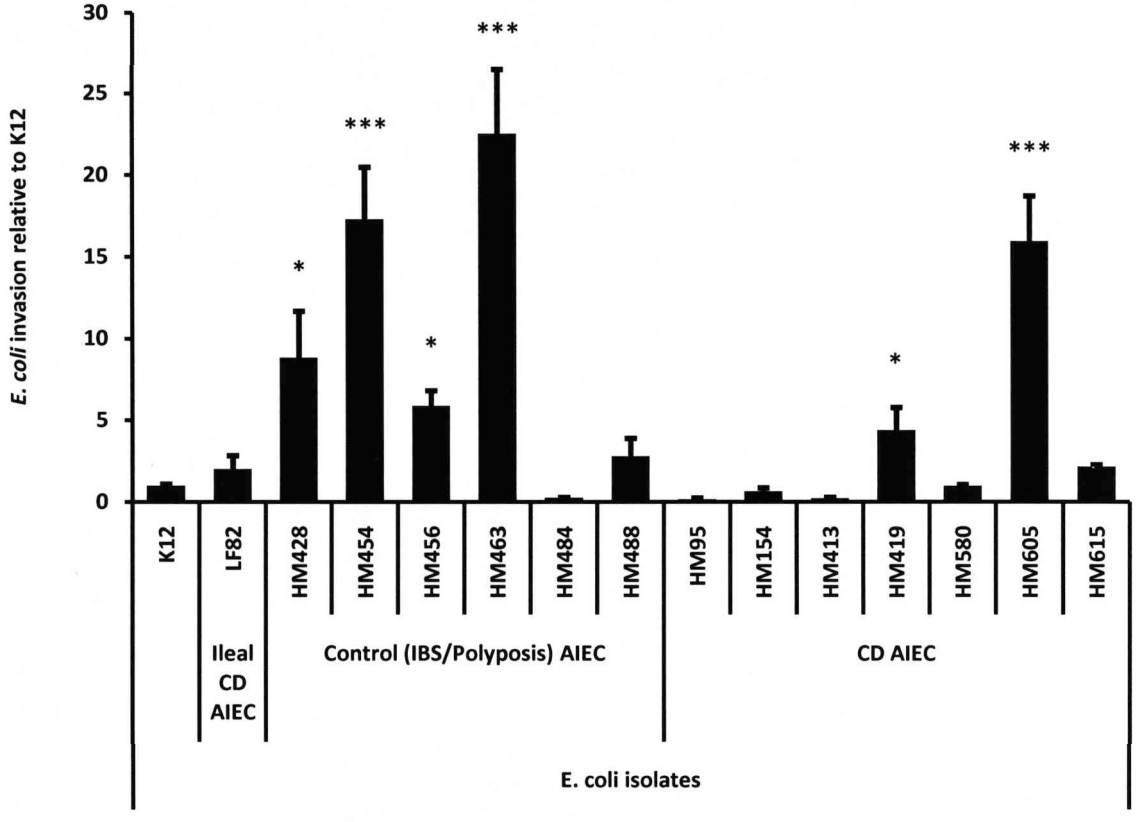


Figure 3.1 AIEC invasion into Caco2 cells

Invasion here is defined as the absolute number of *E. coli* enumerated from within epithelial cells following epithelial cell infection with *E.* coli, gentamicin treatment and cell lysis, and is expressed relative to K12. Significant differences from *E. coli* K12 invasion are as follows: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ (ANOVA) (n=5).

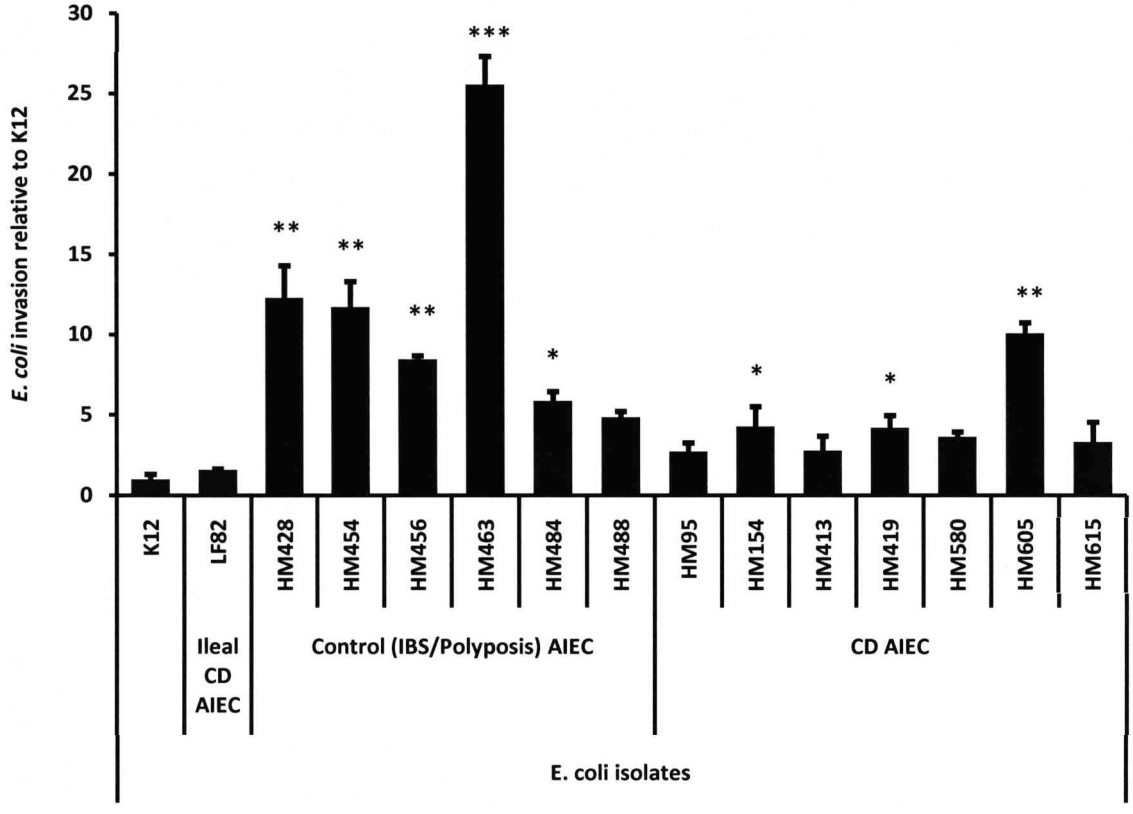


Figure 3.2 AIEC invasion into Caco2-cl1 cells.

Significant differences from *E. coli* K12 invasion are as follows: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ (ANOVA) (n=5)

3.5.2 Bacterial invasion of Caco2-cl1 cells is inhibited by plantain NSP

Plantain NSP at 50 mg / mL was found to significantly inhibit *E. coli* K12 invasion into Caco2-cl1 cells (49.12 ± 15.37% inhibition) (Fig 3.3A). For all of the Crohn's disease AIEC isolates investigated, invasion to Caco2-cl1 cells was inhibited in a dose-dependent fashion (Figure 3.3 B – E); statistically significant inhibition was observed at 50 mg / mL plantain NSP, and also at the lower concentration of 5 mg / mL plantain NSP; inhibition of invasion was also seen at 0.5 mg / mL plantain NSP, although this was not statistically significant. Whilst invasion of all Crohn's disease AIEC isolates into Caco2-cl1 was inhibited by plantain, *E. coli* HM605 and *E. coli* HM615 appeared most sensitive to the presence of plantain, with approximately 90% and 80% of invasion inhibited respectively by plantain at 50 mg / mL.

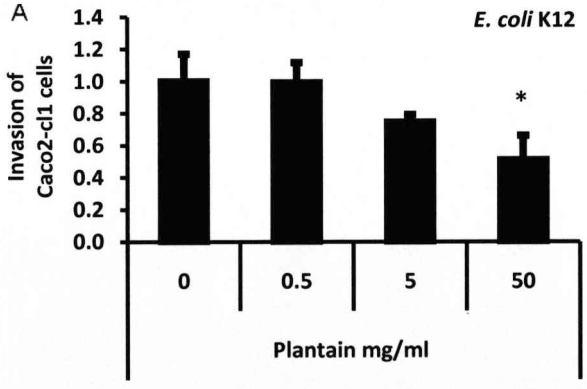


Figure 3.3 (A) – Plantain inhibits bacterial invasion of Caco2-cl1 cells

Invasion of all of the AIEC isolates investigated is inhibited by the presence of plantain NSP at 5 mg / mL and 50mg / mL. For *E. coli* K12, inhibition of invasion is only observed at 50 mg / mL plantain NSP. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ (ANOVA) (n=3)

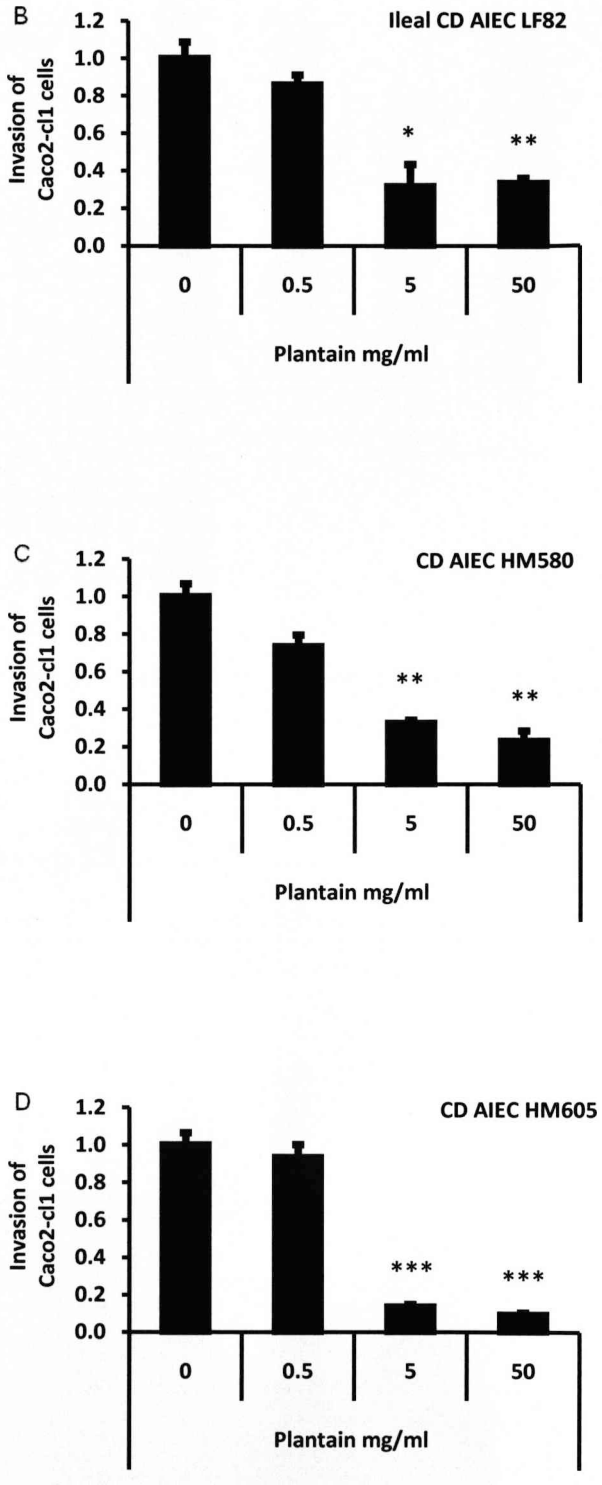


Figure 3.3 (B – D) – Plantain inhibits bacterial invasion of Caco2-cl1 cells

Invasion of all of the AIEC isolates investigated is inhibited by the presence of plantain NSP at 5 mg / mL and 50mg / mL. For *E. coli* K12, inhibition of invasion is only observed at 50 mg / mL plantain NSP. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ (ANOVA) (n=3)

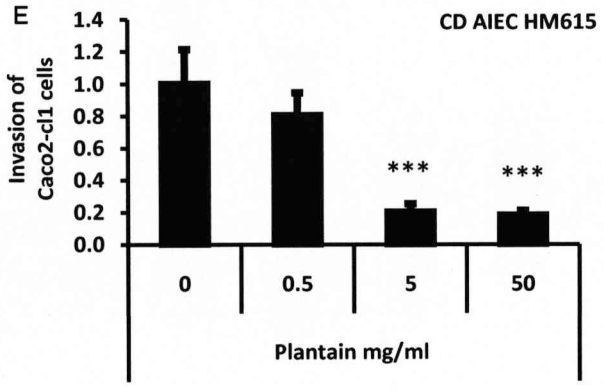


Figure 3.3 (E) – Plantain inhibits bacterial invasion of Caco2-cl1 cells

Invasion of all of the AIEC isolates investigated is inhibited by the presence of plantain NSP at 5 mg / mL and 50mg / mL. For *E. coli* K12, inhibition of invasion is only observed at 50 mg / mL plantain NSP. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ (ANOVA) (n=3)

3.5.3 Bacterial adhesion of Caco2-cl1 cells is inhibited by plantain NSP

The ability of the Crohn's disease AIEC isolates to adhere to the surface of Caco2-cl1 cells was similar to that of *E. coli* K12 – there was no significant difference in levels of adhesion to Caco2-cl1 cells between any of the isolates investigated (Fig 3.4).

Plantain NSP, at 50mg / mL, and 5mg / mL was found to inhibit *E. coli* adhesion to Caco2-cl1 cells (Fig 3.5). At 5 mg / mL plantain, inhibition of adhesion of all *E. coli* strains investigated was in the range of 41 – 46 % inhibition, with the exception of Crohn's disease *E. coli* HM615 which was inhibited by

approximately 60 %. Plantain at 50 mg / mL was found to be more inhibitory of adhesion, with the Liverpool strains (HM---) being inhibited in the region of 65 – 71 %, whilst inhibition of *E. coli* K12 at 48 % was relatively unchanged by the higher plantain concentration, as was AIEC LF82, with an increased inhibition of 1.5 %, despite the 10 fold higher plantain concentration..

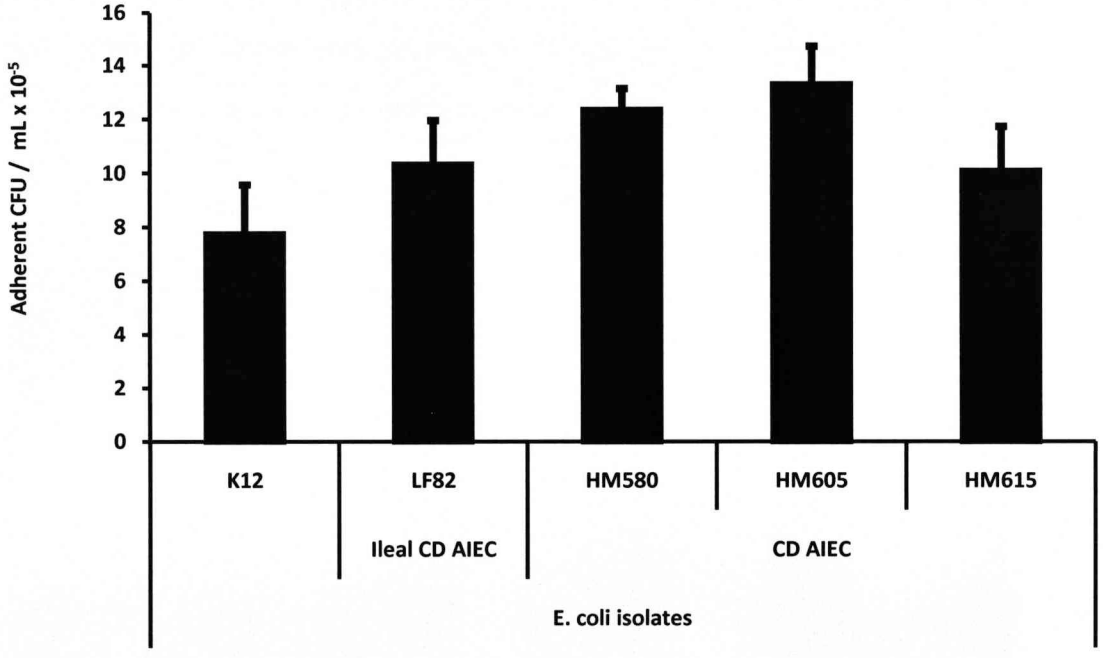


Figure 3.4 *E. coli* isolate adhesion to Caco2-cl1 cells.

Adhesion of *E. coli* K12 is similar to that of the Crohn's disease AIEC isolates investigated (n=3).

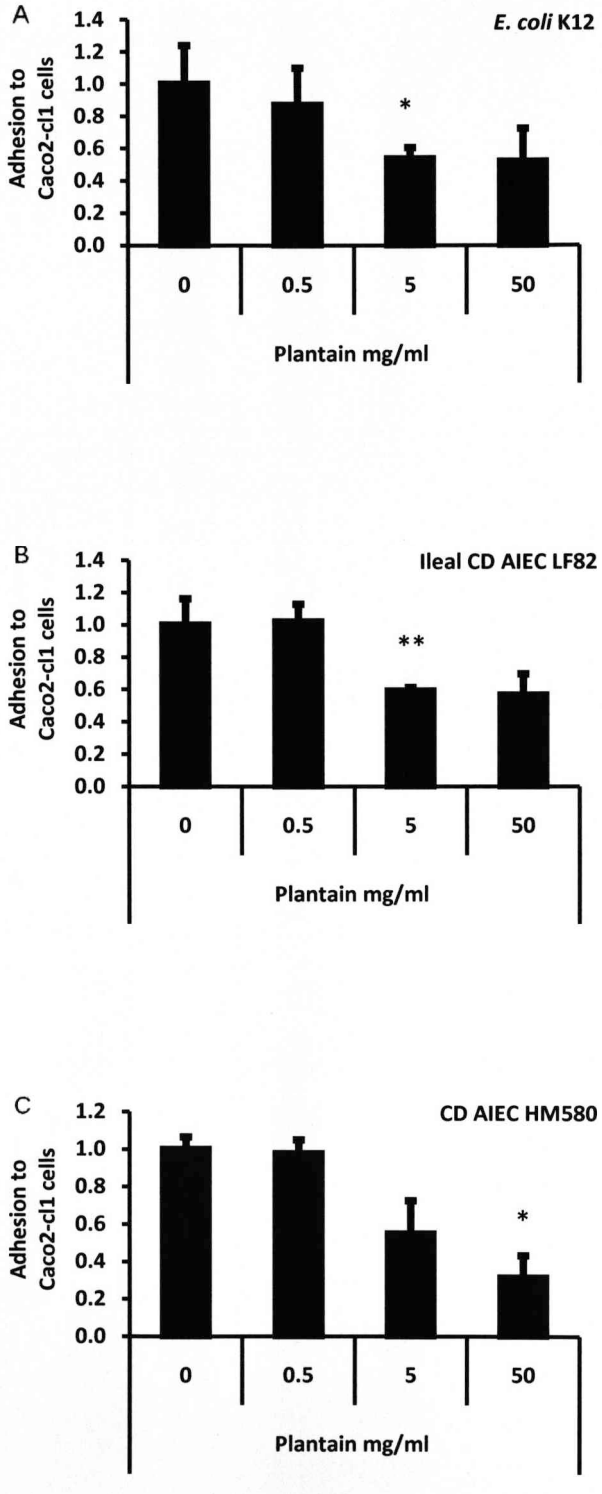


Figure 3.5 (A – C) – Plantain inhibits bacterial adhesion to Caco2-cl1 cells

Adhesion of all of the AIEC isolates investigated and *E. coli* K12 is inhibited by the presence of plantain NSP at 5 mg / mL and 50mg / mL, although not statistically significantly in all cases, despite a 40% inhibition of adhesion. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ (ANOVA) (n=3)

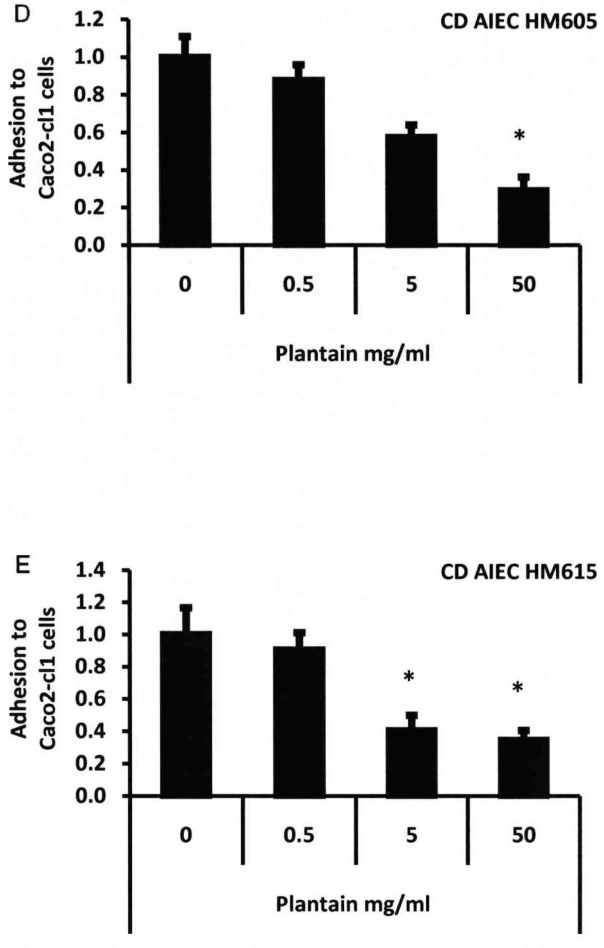


Figure 3.5 (D – E) – Plantain inhibits bacterial adhesion to Caco2-cl1 cells

Adhesion of all of the AIEC isolates investigated and *E. coli* K12 is inhibited by the presence of plantain NSP at 5 mg / mL and 50mg / mL, although not statistically significantly in all cases, despite a 40% inhibition of adhesion. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ (ANOVA) (n=3)

3.6 SUMMARY OF RESULTS

1. Control patient and Crohn's disease AIEC invade into Caco2 and Caco2-cl1 cells to different extents. Invasion does not appear to correlate disease status of the patient from whom the isolates were derived. Some AIEC strains invade Caco2 cells to a lesser extent than control *E. coli* K12; all AIEC invade Caco2-cl1 cells to a greater extent than *E. coli* K12.

2. Plantain NSP at 0.5, 5 and 50 mg / mL inhibits *E. coli* invasion into Caco2-cl1 cells

3. Of the limited number of Crohn's disease AIEC investigated, adherence to Caco2-cl1 cells does not differ to the level of adhesion *E. coli* K12 achieves.

4. Plantain NSP at 5 and 50 mg / mL inhibits *E. coli* adhesion to Caco2-cl1 cells

3.7 DISCUSSION

The initial cause of inflammation in Crohn's disease is unknown. Reports of the increased prevalence of intra-mucosal and mucosa-associated *E. coli* within the tissue of Crohn's disease patients suggest bacterial involvement. These *E. coli* were found in close proximity to the surface of the epithelial lining of the gut, and suggest that microbial-host interactions at this point may be important in the inflammatory process. Indeed, it is possible that bacterial adhesion to, or invasion into host epithelial cells may act as a trigger in the inflammatory response.

The data presented here confirms that of several other published manuscripts [Boudeau *et al.* 1999; Sasaki *et al.* 2007; Eaves-Pyles *et al.* 2008], showing that Crohn's disease *E. coli* isolates are able to adhere to and invade into Caco2 intestinal epithelial cells *in vitro*. The Crohn's disease and control patient *E. coli* isolates described here invade into Caco2 and Caco2-cl1 cells, and adhere to Caco2-cl1 cells.

Co-incubation of Crohn's disease *E. coli* isolates with HT-29 or T84 cells stimulates production of the proinflammatory chemokine Il-8, providing a firm link between Crohn's disease *E. coli* isolates, epithelial cells and the inflammatory response [Subramanian *et al.* 2008]. It is at least a plausible hypothesis that this may be relevant *in vivo*. Indeed, if this is the case, preventing *E. coli* – epithelial cell interaction has the potential to prevent or lessen inflammation which may be of therapeutic benefit. Previous studies have shown that plantain NSP is able to inhibit Crohn's disease *E. coli* isolate adhesion and invasion to I407 cells [Martin *et al.* 2004], and here to Caco2-cl1 cells.

Adherence of diarrheagenic *E. coli* strains (enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adhering *E. coli* (DAEC)) to epithelial cells is a critical early stage in diarrheal infections caused by pathogenic *E. coli* strains. This adherence has been shown to be mediated by lectin-carbohydrate interactions on several independent occasions [Torres *et al.* 2005].

If Crohn's disease *E. coli* isolates also adhere to epithelial cells via lectin-carbohydrate interactions, then it is possible that complex oligosaccharides may interfere with these lectin-carbohydrates interactions, and may result in decreased adherence of Crohn's disease *E. coli* to the epithelia. Soluble plantain NSP is a complex mixture consisting of uronic acids (26.8 %), glucose (25.2 %), mannose (18.7 %) arabinose (9.0 %), galactose (8.6 %), xylose (6.7 %), fucose (2.6%) and rhamnose (2.4 %). It is possible that complex oligosaccharides structures within plantain NSP prevent bacterial – host cell interactions, and a diet supplemented with plantain NSP may be of therapeutic benefit. Boiled green bananas, similar to plantains, were shown in a controlled study in Bangladesh to be effective in a reducing the severity of a variety of diarrhoeal diseases [Rabbani *et al.* 2001].

Despite these *in vitro* findings showing Crohn's disease *E. coli* isolate adhesion and invasion of Caco2 and Caco2-cl1 cells, *in vivo*, *E. coli* have not been observed within normal epithelial cells. In Crohn's disease tissue, the presence of *E. coli* has been most clearly observed within macrophage cells distributed within the lamina propria, beneath ulcers and in granulomas, in the germinal centres of mesenteric lymph nodes, and in giant cells along fissures [Cartun *et al.* 1993; Liu *et al.* 1995; Ryan *et al.* 2004].

It is probably not surprising that *E. coli* have not been observed within normal epithelial cells since *in vivo* accessibility to the epithelial cell surface for bacterial species is somewhat reduced compared with the *in vitro* cell. *In vivo*, intestinal epithelial cells are coated with relatively thick mucus layer which overlies the glycocalyx which consists of a matrix of polysaccharides which serve to prevent bacterial-host cell interaction. Indeed, the *in vivo* epithelial cell surface is normally relatively sterile. This does question the validity of *in vitro* adhesion and invasion data to the *in vivo* situation. It is possible that *in vivo, E. coli* adhesion and invasion of the normal epithelial cells is rare, and that other cells types within the gut may be the main site of interaction for Crohn's disease *E. coli* isolates.

Within the intestine there are a variety of cell types which may potentially interact with AIEC. *In vivo, E. coli* or *E. coli* antigens have been identified within macrophages (by immunohistochemistry [Lui *et al.* 1995] and by laser capture microdissection [Ryan *et al.* 2004]) and within the lamina propria and surrounded inflamed tissue (including ulcers, fissures and in granulomata) [Cartun *et al.* 1993]. More recently, biopsies from Crohn's disease patients were found, following *in vitro* infection with *E. coli* K12 and HB101 to have an accumulation of these *E. coli* within dendritic cells underlying the follicle associated epithelium (FAE) [Keita *et al.* 2008; Salim *et al.* 2009].

This raises the possibility that AIEC may interact with macrophages or dendritic cells *in vivo.* Additionally, since the FAE contains M-cells, which are capable of taking up antigen from the lumen and then delivering it to the intestinal lymphoid system [Kraehenbuhl *et al.* 1992; Neutra *et al.* 1996; Neutra *et al.* 1996], it is possible that AIEC may interact with M-cells *in vivo.* In this way, the M-cells may serve as a route by which the AIEC gain entry to macrophages resident within the lamina propria.

Chapter 4

Modelling M-cells *In Vitro*, and Bacterial Translocation across this Specialised Epithelium

4.1 HYPOTHESIS

It is possible to model, *in vitro*, cells which structurally and functionally resemble intestinal M-cells. Translocation of Crohn's disease *E. coli* isolates will be increased across these M-cells compared with translocation across control Caco2-cl1 cells.

4.2 AIMS

1. To model intestinal M-cells of the follicle-associated epithelium (FAE) *in vitro*, demonstrating biochemically, functionally and structurally that they are M-cells
2. To establish and characterise translocation of Crohn's disease *E. coli* isolates across these *in vitro* M-cells

4.3 INTRODUCTION

Aphthoid lesions of Crohn's Disease mainly occur within the follicle-associated epithelium (FAE) of the gut [Morson 1972; Rickert *et al.* 1980; Fujimura *et al.* 1996]. In Crohn's disease of the large bowel, aphthoid lesions frequently occur over lymphoid aggregates within the FAE [Fujimura *et al.* 1996], whilst in Crohn's disease of the small bowel, these lesions typically occur over Peyer's patches [Rickert *et al.* 1980; Rutgeerts *et al.* 1984; Olaison *et al.* 1992; Shikuwa *et al.* 2007], structured collections of lymphoid follicles within the FAE [Van Kruiningen *et al.* 2002].

The FAE overlies the mucosa-associated lymphoid tissue (MALT), situated just below the epithelium, and is the location of M-cells ('membranous' or 'microfold' cells) [Owen *et al.* 1974]. These M-cells are the major site of both antigen and microorganism sampling in the gut – they are able to internalise macromolecules and microorganisms, and deliver them to the underlying lymphoid tissue, providing a portal from which an immune response can be mounted [Neutra 1999; Gullberg *et al.* 2000]. They are a major site for pathogen invasion and uptake, including *Salmonella* Typhimurium and *Shigella* spp. [Siebers *et al.* 1996; Jensen *et al.* 1998; Jepson *et al.* 2001]. Since the initial lesions observed in Crohn's disease occur at Peyer's patches it is possible that AIEC, like *Salmonella* spp. and *Shigella* spp., are translocated by intestinal M-cells and subsequently delivered to the underlying macrophage cells, where they have been observed *in vivo* [Liu *et al.* 1995; Ryan *et al.* 2004].

4.4 METHODS

Detailed methods can be found in chapter 2. Briefly, the reproducible generation of M-cell cell enriched monolayers from Caco2-cl1 cell monolayers was obtained by growth of Caco2-cl1 cells for 14 – 16 days on Transwell® filters, followed by 6 days of basolateral co-culture with Raji B cells beneath the Transwell® filters. In this way, M-cell enriched monolayers were generated, with maintenance of monolayer integrity assessed through monitoring transepithelial electrical resistance (TEER).

Following successful M-cell generation, M-cells and control Caco2-cl1 cells were infected apically with various bacterial species to assess translocation across M-cell monolayers and Caco2-cl1 monolayers. Bacterial species

utilised in this study were *Salmonella* Typhimurium LT2, *Shigella sonnei*, control *E. coli* K12, *E. coli* XL1-Blue, probiotic *E. coli* Nissle 1917, the classical French ileal AIEC LF82, seven Liverpool Crohn's disease *E. coli* isolates, and five Liverpool control subject *E. coli* isolates. To assess translocation, bacterial species were added apically to Transwells® at a multiplicity of infection (MOI) of 10 (10 bacteria added per cell); following a 4 hour infection, basolateral media containing translocated bacteria was serially diluted, plated onto LB agar in triplicate, and grown overnight at 37°C. Colony forming units (CFU), assumed to originate from a single, viable, translocated bacteria were then counted, and translocated expressed per cm^2 epithelial monolayer. (M-cell monolayers which had been generated by Raji B co-culture for 4 – 8 days (rather than the optimal 6 days) were also investigated for their ability to translocate bacterial species using this method).

Assays to establish the time-course of translocation across both M-cells and Caco2-cl1 cells were conducted in the same way using the Liverpool Crohn's disease *E. coli* isolate HM605 – aliquots of the basolateral media were removed at set times post infection, and translocated CFU quantified.

The method by which *E. coli* HM605 was grown was investigated in relation to translocation across M-cells. HM605 was grown by overnight culture on solid LB agar, by overnight culture in liquid LB broth, or by overnight culture in liquid LB broth followed by 48 h suspension in sterile PBS. Following apical infection of the M-cell monolayers at an MOI of 10, aliquots of the basolateral media were removed and translocated CFU quantified.

In addition, Caco2-cl1 and M-cell monolayers were processed for transmission electron microscopy (TEM) analysis. This was to allow examination of the cell structure of the monolayers, particularly the distribution of microvilli on the apical cell surface. TEM analysis was also used to examine the presence of bacterial species within the cells of the monolayer.

Western blot analysis was conducted to determine expression levels of alkaline phosphatase, previously reported to be down-regulated in M-cells [Brown *et al.* 1990; Clark *et al.* 1994; Tyrer *et al.* 2002]. *Aleuria aurantia* lectin binding was also investigated as it had been previously reported to be able to selectively target M-cells [Roth-Walter *et al.* 2005].

4.5 RESULTS

4.5.1 TEER increases during Caco2-cl1 and M-cell monolayer formation

In the absence of a thin Matrigel™ layer on the Transwell® filter, background resistance readings were in the range of 40 – 50 Ωcm^2; following addition of Matrigel™, background resistance readings were in the range of 66 - 78 Ωcm^2; monolayer TEER was calculated as the additional TEER generated by the presence of Caco2-cl1 cells above this Matrigel™ background.

During the course of Caco2-cl1 cell culture, monolayer TEER steadily increased (Figure 4.1). During days 0 to 14 – 16, monolayer TEER typically increased from 0 Ωcm^2 to 300 Ωcm^2, indicating the generation of fully confluent differentiated monolayers. Only those monolayers which had TEER values in excess of 300 Ωcm^2 after 16 days of growth were used. Caco2-cl1 monolayers which were co-cultured with Raji B cells during the following 4 – 6 days formed M-cell monolayers. Those without Raji B co-culture formed Caco2-cl1 monolayers. In both cases, TEER continued to increase (Figure 4.1). At the end of culture, typically 20 – 22 days, Caco2-cl1 monolayers had slightly higher TEER values than M-cell monolayers. In an assay by assay basis, this difference was always present, although it was typically less than 50 Ωcm^2.

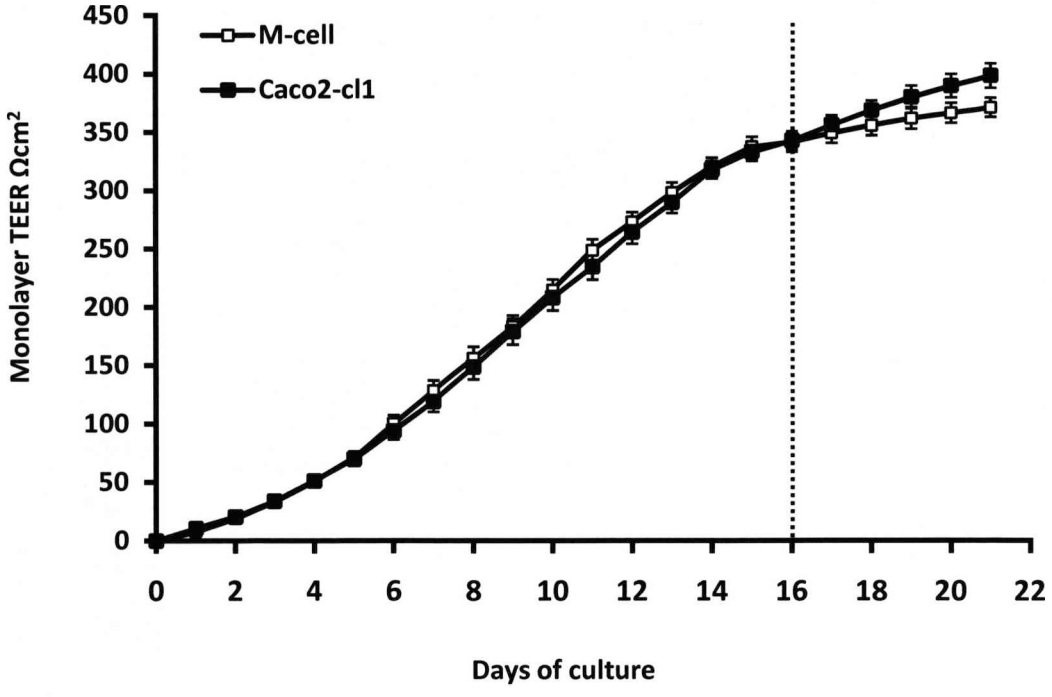
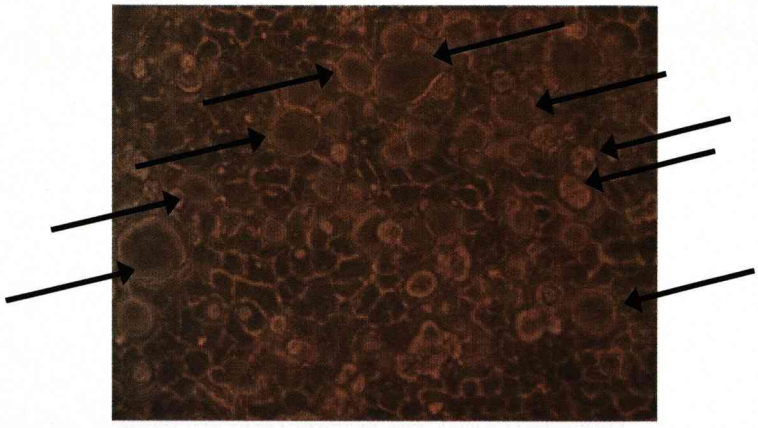


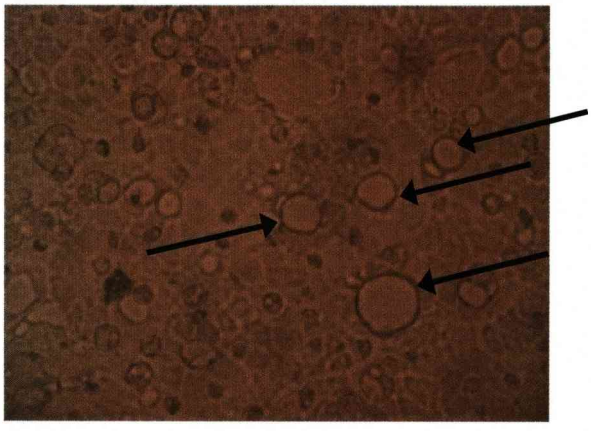
Figure 4.1 A typical production of *in vitro* derived M-cells.

Monolayer TEER steadily increases during culture, indicating formation of fully confluent, differentiated monolayers (TEER values in excess of 300 Ωcm^2). Monolayer incubation with Raji B cells occurred on day 16 (dashed line) and continued for the following five days, leading to the formation of M-cells, whilst control Caco2-cl1 monolayers formed in the absence of Raji B cells. The TEER of the control Caco2-cl1 monolayers continued to increase over the following five days of incubation (days 16 – 21; closed boxes), as did the TEER of the M-cells (open boxes), although to a lesser extent. (Data represents n = 35 culture Transwells® for each group (Caco2-cl1 and M-cell))

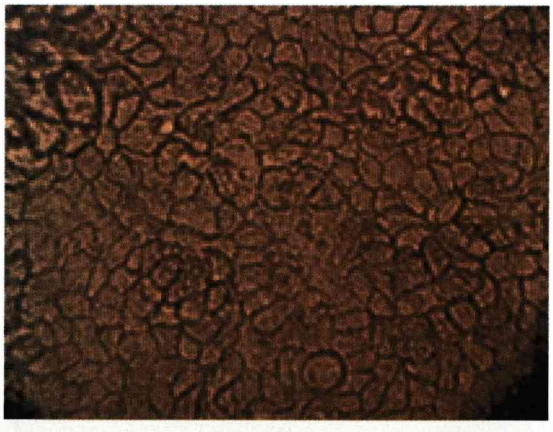
In addition to TEER, monolayer integrity was also monitored visually. During early culture, typically days 0 – 5, the monolayer could clearly be seen to have numerous gaps within it (Figure 4.2A). By day 8, these gaps within the monolayer persisted, although were much fewer in number, implying that the gaps had been filled through cell growth and cell division (Figure 4.2B). By day 11, the gaps were no longer visible by light microscopy (Figure 4.2C).



A ~ 40 Ωcm^2 (day 3)



B ~ 130 Ωcm^2 (day 7)



C ~ 220 Ωcm^2 (day 11)

Figure 4.2 TEER increases as cell confluency increases

(A) During the first days of culture, various gaps between Caco2-cl1 cells were visible (black arrows). (B) During days 5 - 9, fewer gaps between Caco2-cl1 cells were visible (black arrows), and there was an increase in monolayer TEER. (C) After day eleven of culture, visibly intact Caco2-cl1 monolayers are observed, and gaps between cells could not be detected. Again there was an increase in TEER.

4.5.2 *In vitro* generated M-cells have a characteristic M-cell structure

Following culture of Caco2-cl1 and M-cell monolayers, transmission electron microscopy (TEM) revealed the M-cells to be structurally different from Caco2-cl1 cells in their microvilli distribution. All of the Caco2-cl1 monolayer examined had homogeneous microvilli expression on the apical surface of the cells; microvilli appeared to be uniform length, and of uniform distribution across the apical cell surface (Figure 4.3A). In contrast, M-cell monolayers were characterised by both regions containing apical cell surface microvilli expression, and regions absent of microvilli expression (Figure 4.3B). This was characteristic of all cultures (n = 3) which were examined by TEM. High-magnification images of the apical cell surface of these representative M-cell productions can be seen in Figure 4.4.

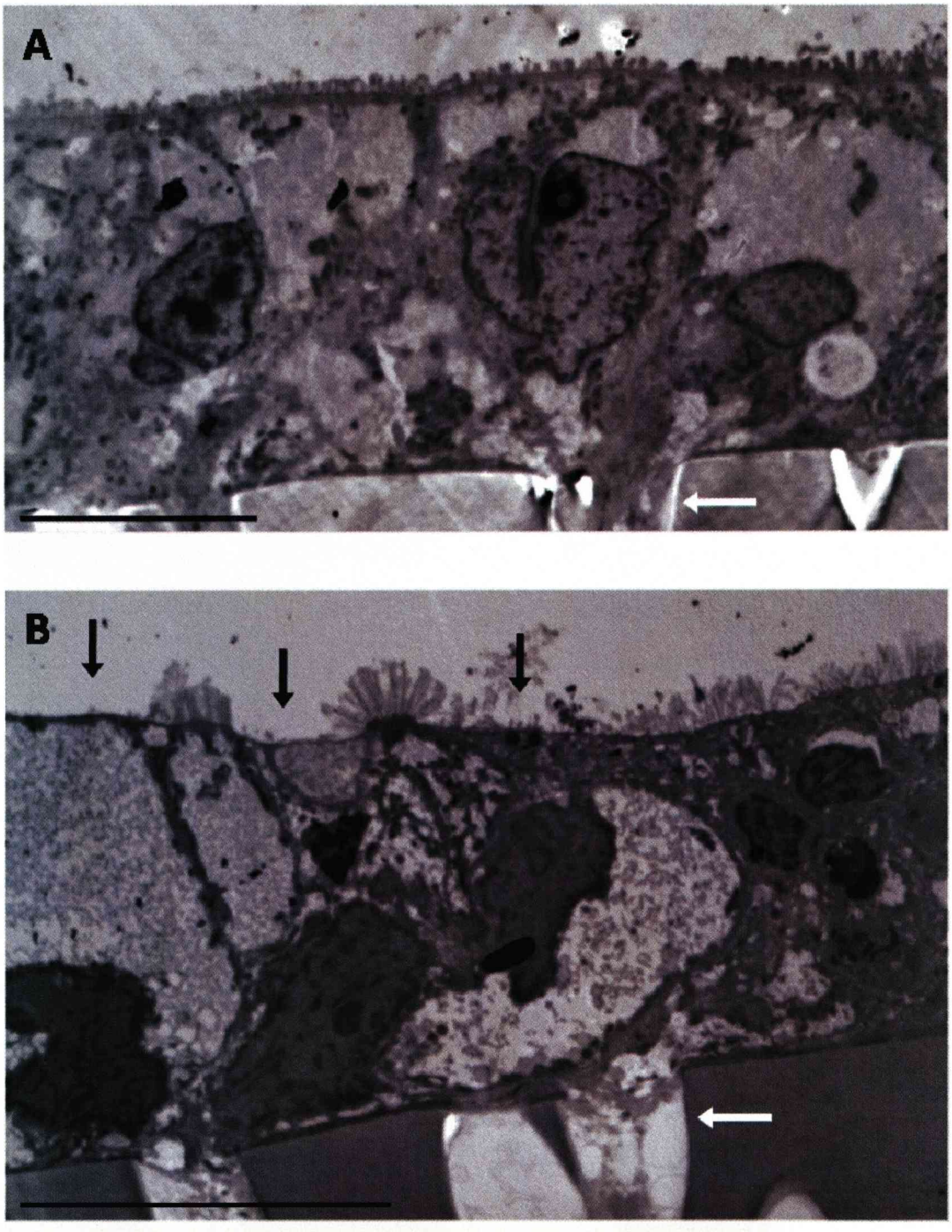


Figure 4.3 TEM reveals structural differences between Caco2-cl1 and M-cell monolayers

(A) TEM image of a Caco2-cl1 monolayer. Closely packed microvilli can clearly been seen on the apical aspect of the cells. (B) TEM image of an M-cell monolayer. The apical surface of the cells is heterogeneous for microvilli expression; lack of microvilli expression is indicated by black arrows. In both images, the Transwell® filter support can be seen on the lower portion of the image, as can the 3μm pores within the filter (indicated by white arrows). Bar = 10μm

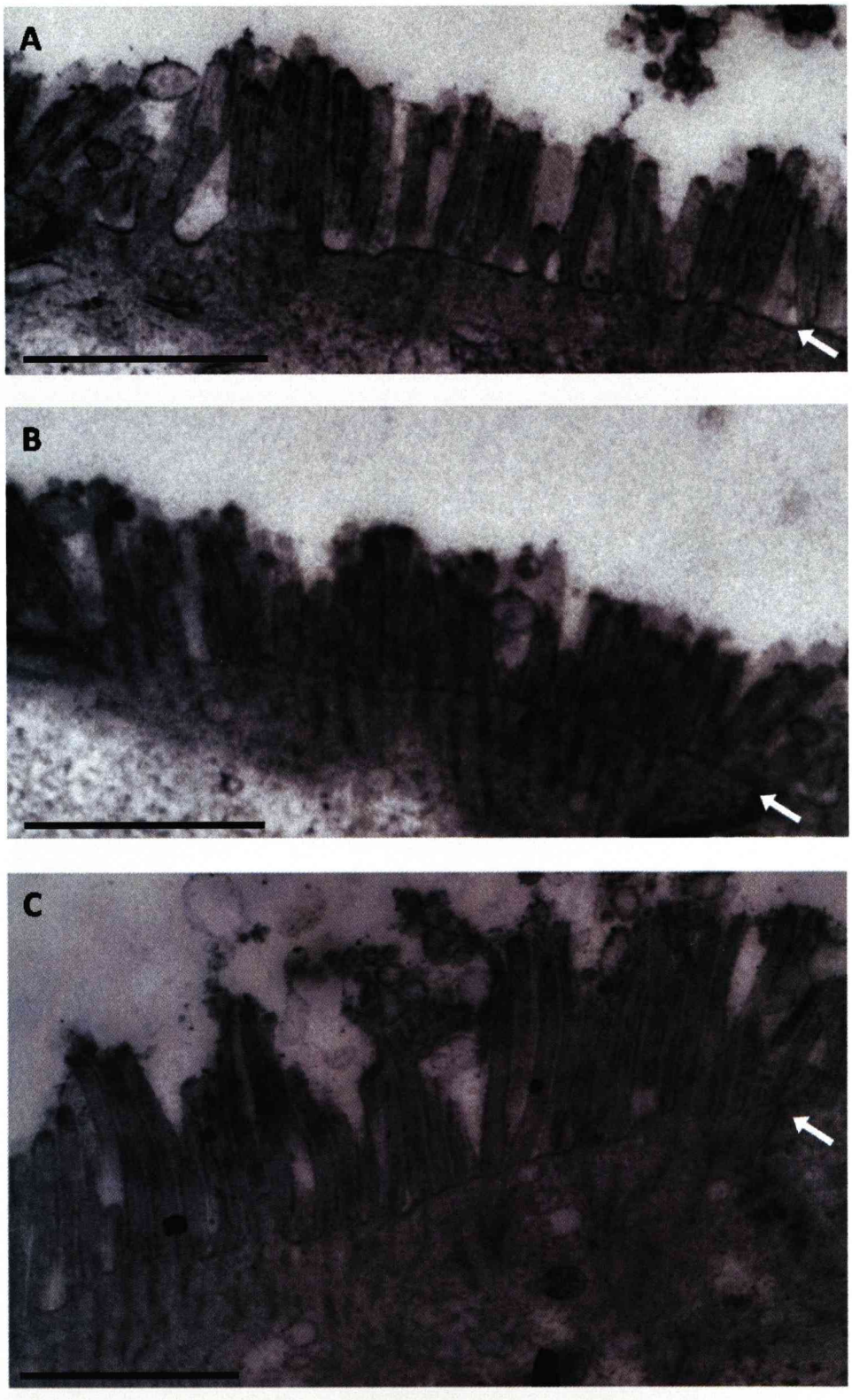


Figure 4.4 (A – C) – Apical surfaces of Caco2-cl1 and M-cells

(A – C) TEM images of the apical surface of Caco2-cl1 monolayers, showing closely packed microvilli at the apical cell surface (white arrows). Bar = 1µm

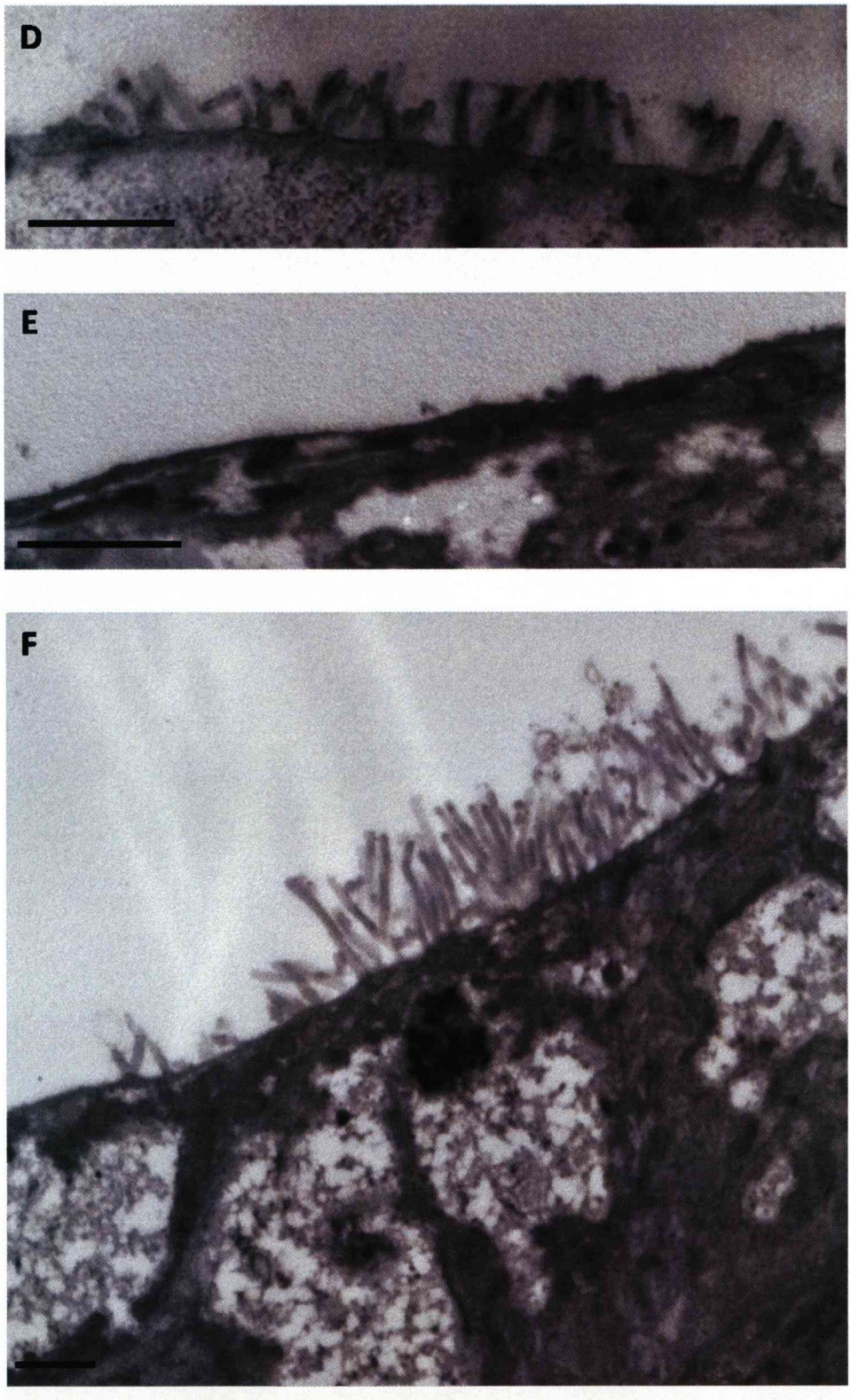


Figure 4.4 (D – F) – Apical surfaces of Caco2-cl1 and M-cells

(D – J) TEM reveals a large variation in M-cell apical surface. Bar = 1µm

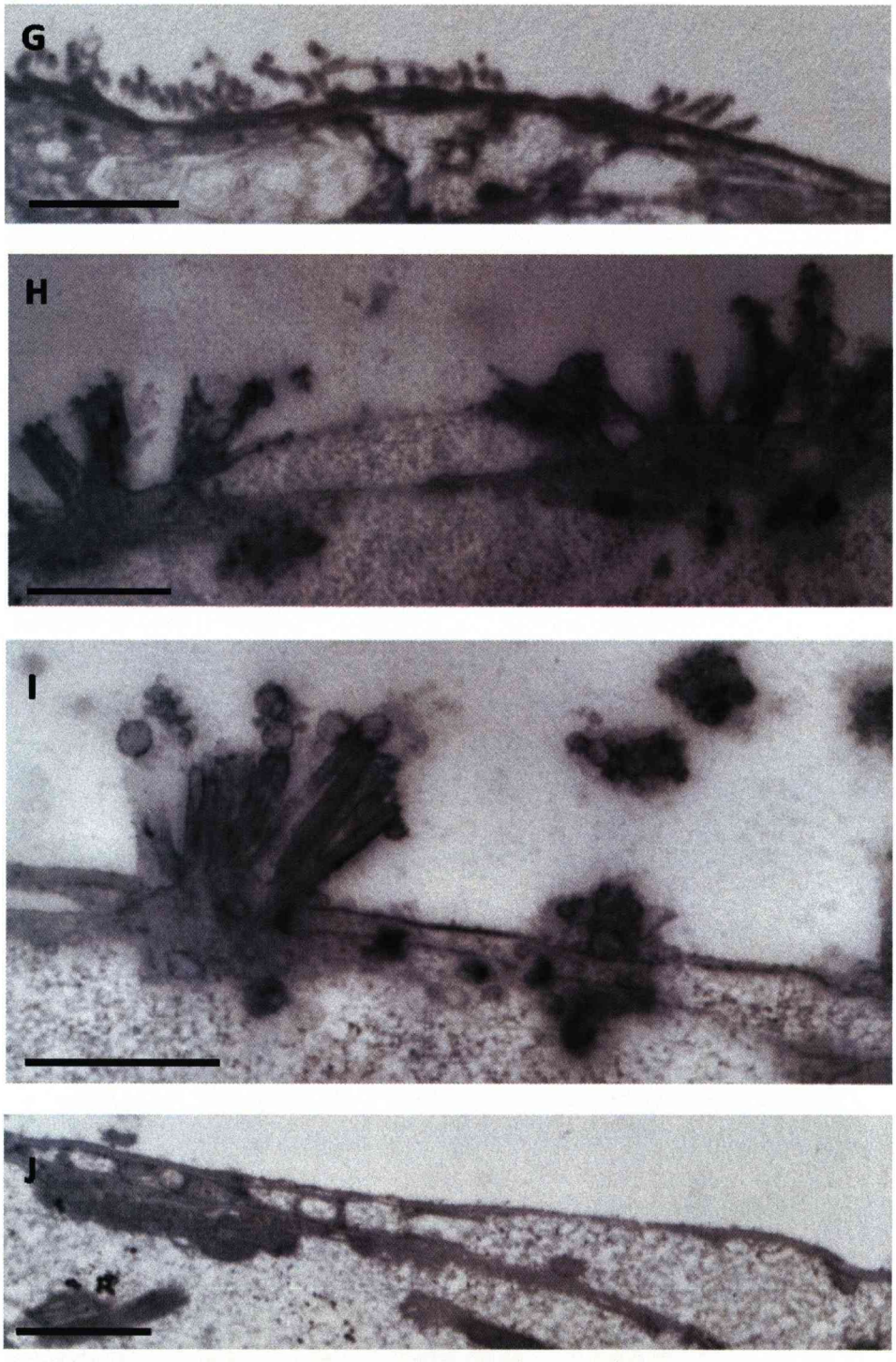


Figure 4.4 (G – J) – Apical surfaces of Caco2-cl1 and M-cells

(D – J) TEM reveals a large variation in M-cell apical surface. Bar = 1μm

4.5.3 *In vitro* generated M-cells display biochemical variations compared to Caco2-cl1 cells

Western blotting revealed that there was an increase in *Aleuria aurantia* lectin (AAL) total binding to M-cell lysates compared with total binding to Caco2-cl1 cell lysates (Figure 4.5); densitometry analysis of Western blots revealed this increase to be approximately 15.4 ± 1.5 % in M-cells compared to Caco2-cl1 monolayers (n = 4). Binding of AAL appears non-specific, as can be seen from the diverse number of bands of binding of different molecular sizes. There does however seem to be an increase of AAL binding to proteins with a higher molecular weight than smaller proteins. AAL binds to Fucα1-2Galβ1-4(Fucα1-3/4)Galβ1-4GlcNAc and R_2-GlcNAcβ1-4(Fucα1-6)GlcNAc-R_1; heavily fucosylated proteins may not migrate on the SDS-PAGE gel as effectively as non fucosylated proteins, giving them a falsely high protein molecular weight – thus perhaps accounting for the increase of AAL binding of higher molecular weight proteins.

Alkaline phosphatase expression was also investigated. It was found that M-cell monolayers had a lower total expression of alkaline phosphatise than Caco2-cl1 monolayers (Figure 4.6); densitometry analysis of Western blots revealed this decrease to be approximately 21.6 ± 4.1 % in M-cells compared to Caco2-cl1 monolayers (n = 4)

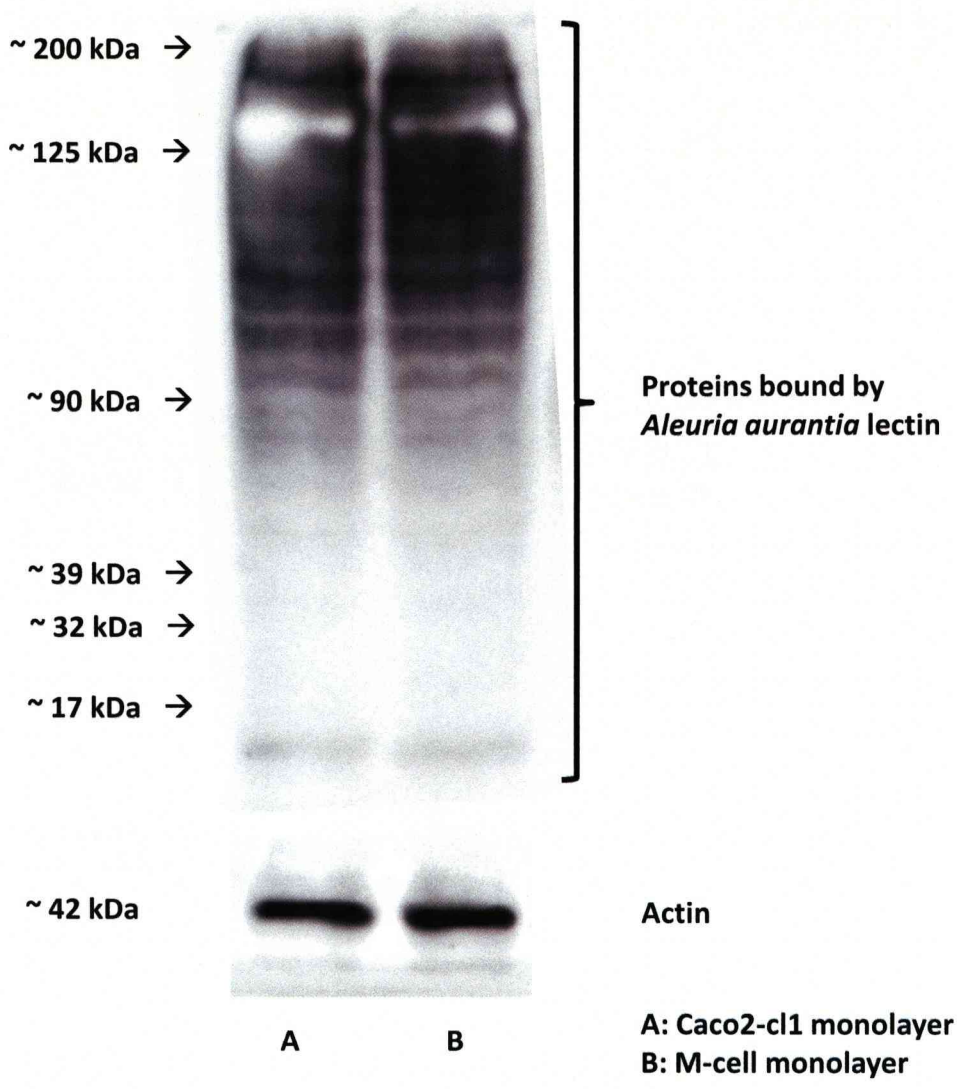


Figure 4.5 *Aleuria aurantia* lectin binding

Representative Western blot showing an increase in *Aleuria aurantia* lectin binding to M-cell monolayer cell lysates compared to Caco2-cl1 cell monolayer lysates (representative of 4 individual M-cell productions).

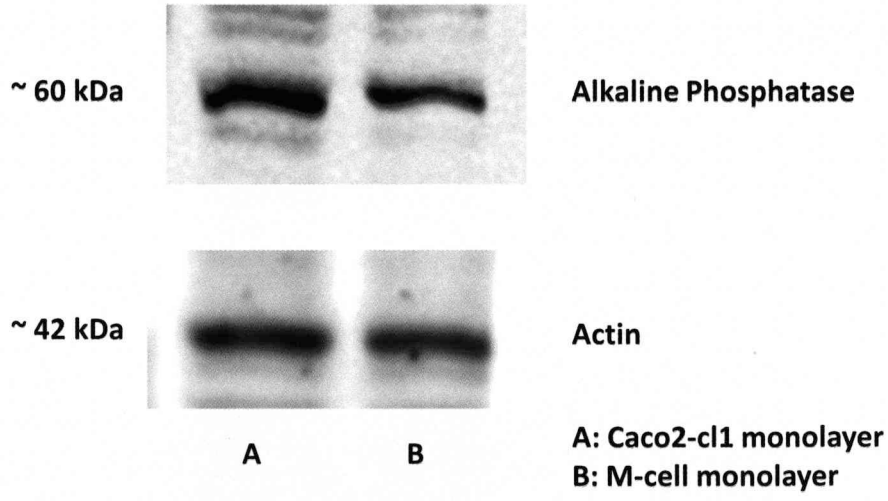


Figure 4.6 Alkaline Phosphatase expression

Representative Western blot showing a decrease of alkaline phosphatase protein expression in M-cell monolayers compared to Caco2-cl1 cell monolayers (representative of 4 individual M-cell productions).

4.5.4 Translocation of *Salmonella* Typhimurium LT2 is increased across M-cell monolayers

Translocation of *Salmonella* Typhimurium LT2 from the apical side to the basolateral side of the monolayer was established. Translocation through M-cell monolayers was significantly higher than translocation through Caco2-cl1 monolayers following a 4 hour infection (Figure 4.7).

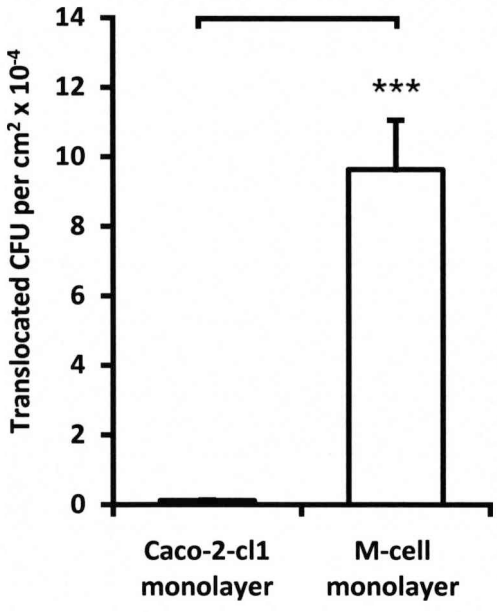


Figure 4.7 *Salmonella* Typhimurium LT2 translocates through *in vitro* M-cells

Translocation of *Salmonella* Typhimurium LT2 was approximately 80 fold higher than through Caco2-cl1 cells. ***, $P < 0.001$ (ANOVA) (n = 3)

Monolayer TEER decreased slightly in response to infection with *Salmonella* Typhimurium LT2 (Figure 4.8). After a 4 hour infection with *S.* Typhimurium LT2, Caco2-cl1 monolayer TEER decreased by 8.91 ± 1.47 %, whilst M-cell monolayer TEER decreased by 11.96 ± 4.89 %.

Translocation of *Salmonella* Typhimurium LT2 across an individual well was found not to correlate to the TEER of that well (Figure 4.9). In each assay conducted, there is overlap between Caco2-cl1 monolayer TEER and the M-cell TEER; the highest translocation of CFUs was always seen across M-cell monolayers. This however did not always correlate to wells with the lowest TEER values, indicating that TEER was not a direct measure of translocation, and that lower TEER values do not absolutely correlate higher translocation levels.

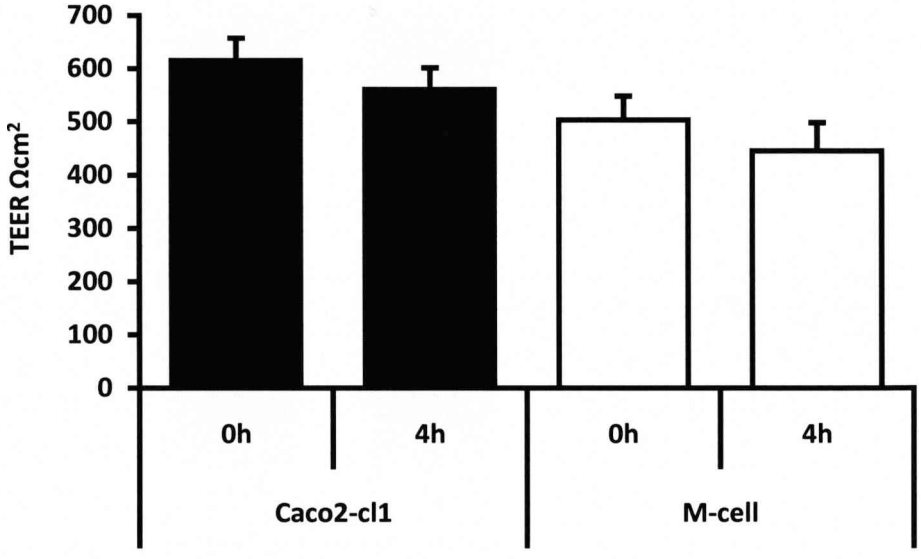


Figure 4.8 TEER decreases in response to *Salmonella* Typhimurium LT2

Monolayer TEER for both Caco2-cl1 monolayers and M-cells monolayers decreased during the course of a 4 hour *Salmonella* Typhimurium LT2 infection, although the decrease was not statistically significant. (n = 5)

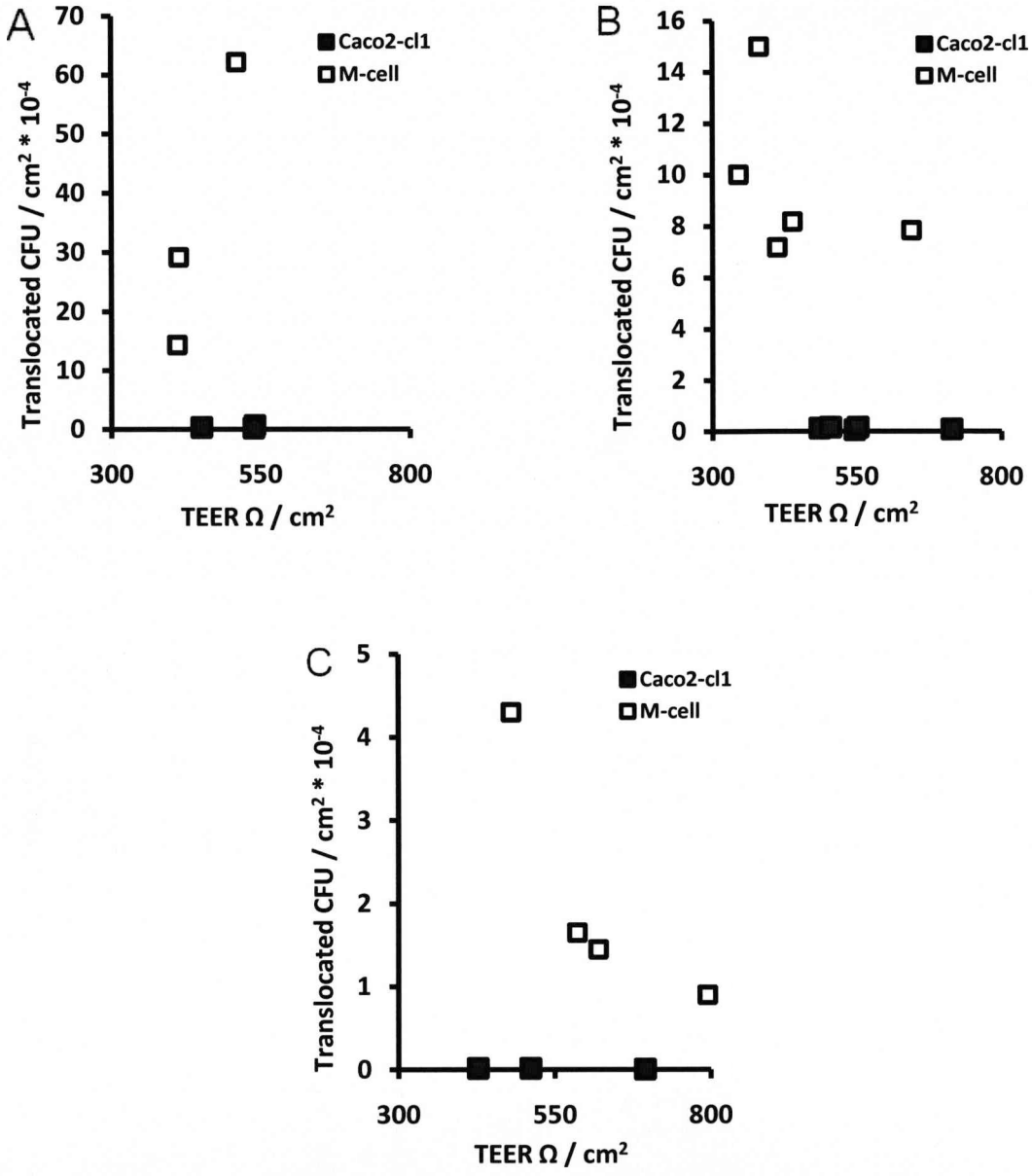


Figure 4.9 TEER and Translocation of *Salmonella* Typhimurium LT2

A, B and C show three individual assays to assess *Salmonella* Typhimurium LT2 translocation across Caco2-cl1 and M-cell monolayers – they indicate that translocation of *Salmonella* Typhimurium LT2 was independent of the TEER of the monolayer following a 4 hour infection (multiplicity of infection (MOI) = 10).

4.5.5 Translocation of *Shigella sonnei* is increased across M-cell monolayers

As with *Salmonella* Typhimurium LT2, translocation of *Shigella sonnei* is increased across M-cell monolayers compared to Caco2-cl1 monolayers by approximately 8 fold (Figure 4.10). Again, this increase in translocation was not connected to the TEER of the monolayer (Figure 4.11).

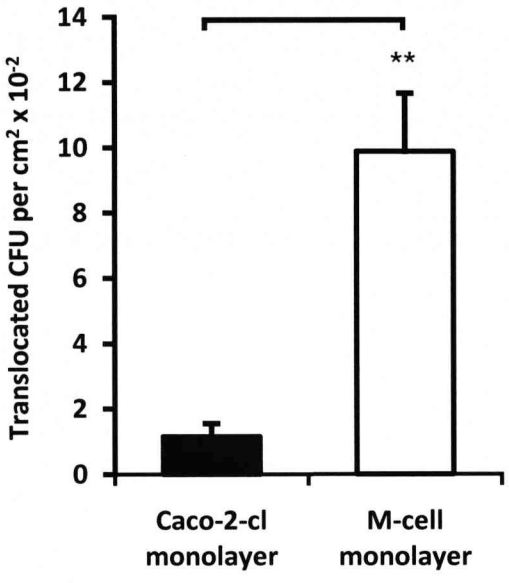


Figure 4.10 *Shigella sonnei* translocates through *in vitro* M-cells

Translocation of *Shigella sonnei* was approximately 8 fold higher than through Caco2-cl1 cells. **, $P < 0.01$ (ANOVA) (n = 3)

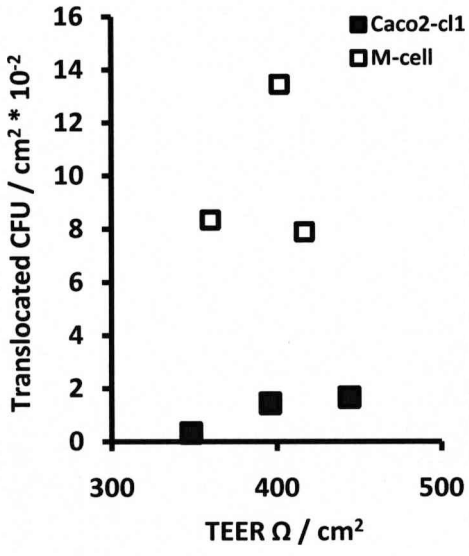


Figure 4.11 TEER and Translocation of *Shigella sonnei*

An individual assay to assess *Shigella sonnei* translocation across Caco2-cl1 and M-cell monolayers – it indicates that translocation of *Shigella sonnei* LT2 was independent of the TEER of the monolayer after a 4 hour infection (MOI = 10).

4.5.6 Translocation of control *E. coli* K12, *E. coli* XL1-Blue and probiotic *E.coli* Nissle 1917 are not increased across M-cell monolayers

Unlike *Salmonella* Typhimurium LT2, translocation of control *E. coli* K12, *E. coli* XL1-Blue and probiotic *E. coli* Nissle 1917 are not significantly increased across M-cell monolayers compared to Caco2-cl1 monolayers (Figure 4.11). Translocation of these strains did not affect TEER during infection, with a less than 5 % change in TEER for each strain during a 4 hour infection.

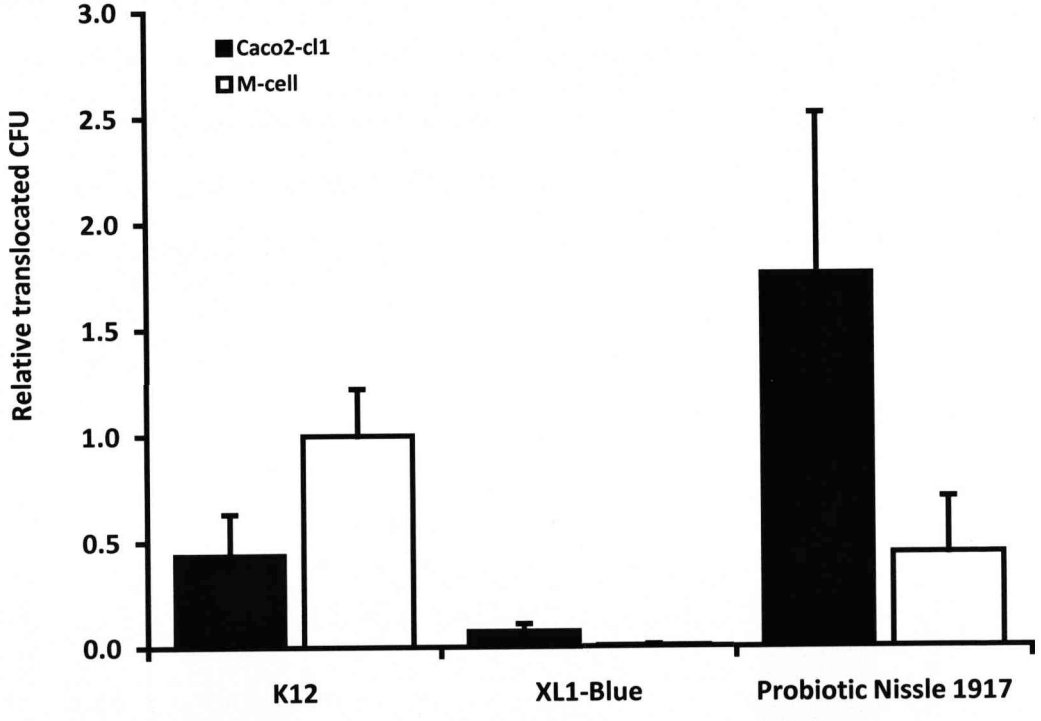


Figure 4.11 Translocation of control *E. coli* across Caco2-cl1 and M-cell monolayers

E. coli K12 does not significantly translocate through *in vitro* M-cells any more so than through Caco2-cl1 cells (n = 8). The laboratory *E. coli* strain XL1-Blue does not appear to be translocated through *in vitro* M-cells (n=3), whilst numerically, translocation of Probiotic Nissle 1917 was reduced through M-cells compared to its translocation through Caco2-cl1 cells. The difference in translocation across Caco2-cl and M-cell monolayers for each control *E. coli* strain was not significant (ANOVA) (n=3)

4.5.7 Characterising the M-cell model using Crohn's disease *E. coli* HM605 - effect of TEER upon translocation

Crohn's disease AIEC HM605 was used initially to establish experimental conditions to investigate translocation across M-cells. Both Caco2-cl1 and M-cell monolayers were generated with TEER in the range of 0 – 800 Ωcm^2 and 0 – 450 Ωcm^2 respectively. Following a 5 minute infection with HM605, monolayers with TEER values < 100 Ωcm^2 showed in excess of 10% of the initial HM605 inoculum passing through both Caco2-cl1 and M-cell monolayers (equivalent to ~ 1.6 x 10^6 CFU / cm^2). Both Caco2-cl1 and M-cell monolayers with TEER > 180 Ωcm^2 did not show any translocation of HM605 during this 5 minute infection (Figure 4.12A). There is a very distinct gap between those monolayers which do not translocate HM605 within a 5 minute infection, and those that do, which appears closely linked to the monolayer TEER.

Following a 30 minute infection, both Caco2-cl1 and M-cell monolayers with TEER < 200 Ωcm^2 were found to have HM605 within the basolateral compartment, with approximately 1 – 10 % of the initial inoculum having transferred from the apical to the basolateral chamber. Those monolayers with TEER > 200 Ωcm^2 showed limited transfer of HM605 from the apical to basolateral chamber (M-cell monolayer, 0.04 ± 0.06 %; Caco2-cl1 monolayer, 0.005 ± 0.009 %) (Figure 4.12B). These findings confirm the decision to regard TEER > 300 Ωcm^2 as being fully confluent monolayers.

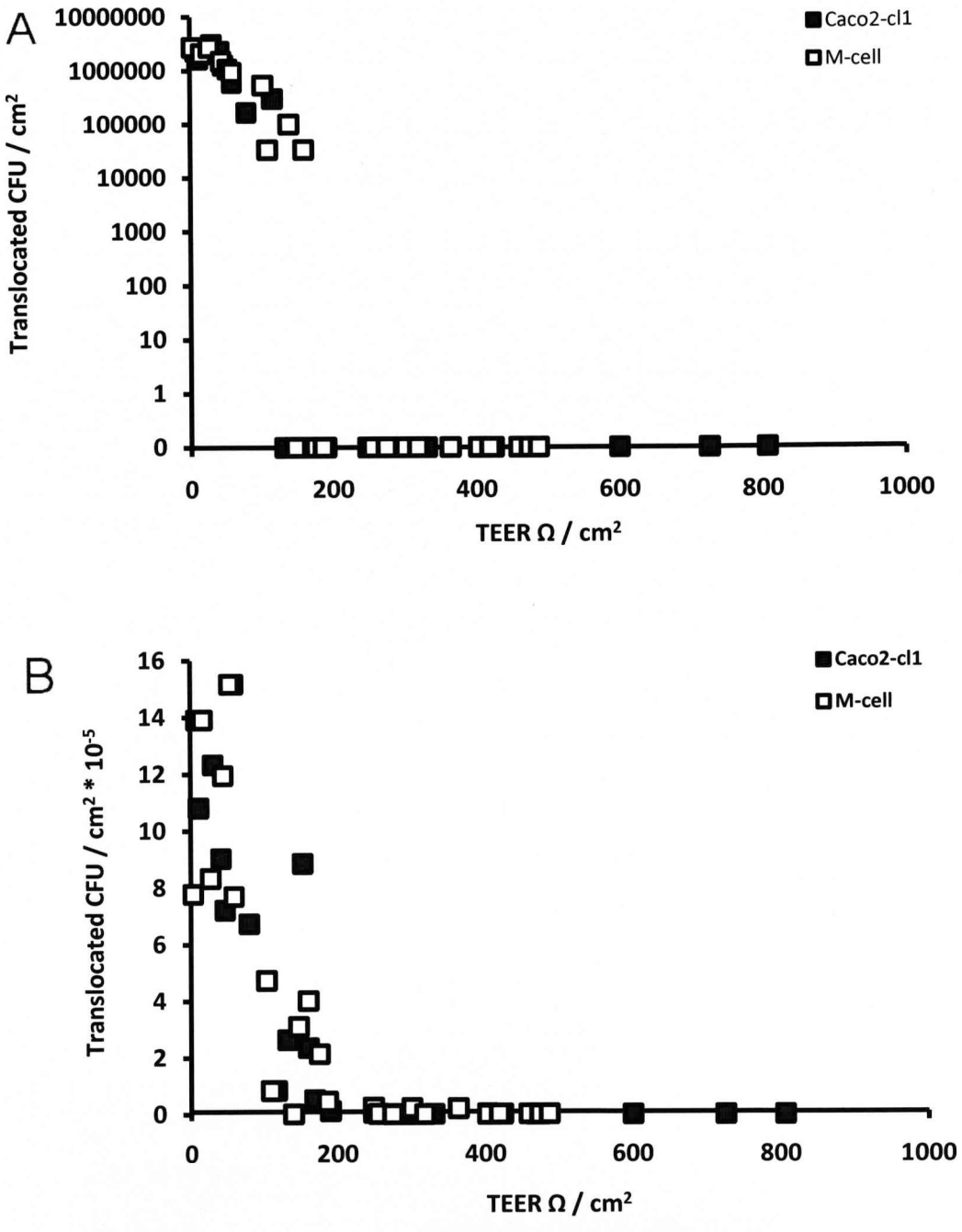


Figure 4.12 TEER and translocation of Crohn's disease AIEC HM605

Passage of Crohn's disease AIEC HM605 across Caco2-cl1 and M-cell monolayers following a (A) 5 minute infection and (B) a 30 minute infection.

4.5.8 M-cell translocation is affected by the length Raji B cell co-culture with Caco2-cl1 monolayers

During the first six days of co-culture of Caco2-cl1 monolayers with Raji B cells to generate M-cells, the TEER of the monolayer increases slightly, although to a lesser extent than the TEER of control Caco2-cl1 monolayers. By the eighth day of co-culture, the TEER of the M-cell monolayers falls significantly to below 300 Ωcm^2, whilst the TEER of Caco2-cl1 monolayers remains, on average, in excess of 600 Ωcm^2 (Figure 4.13).

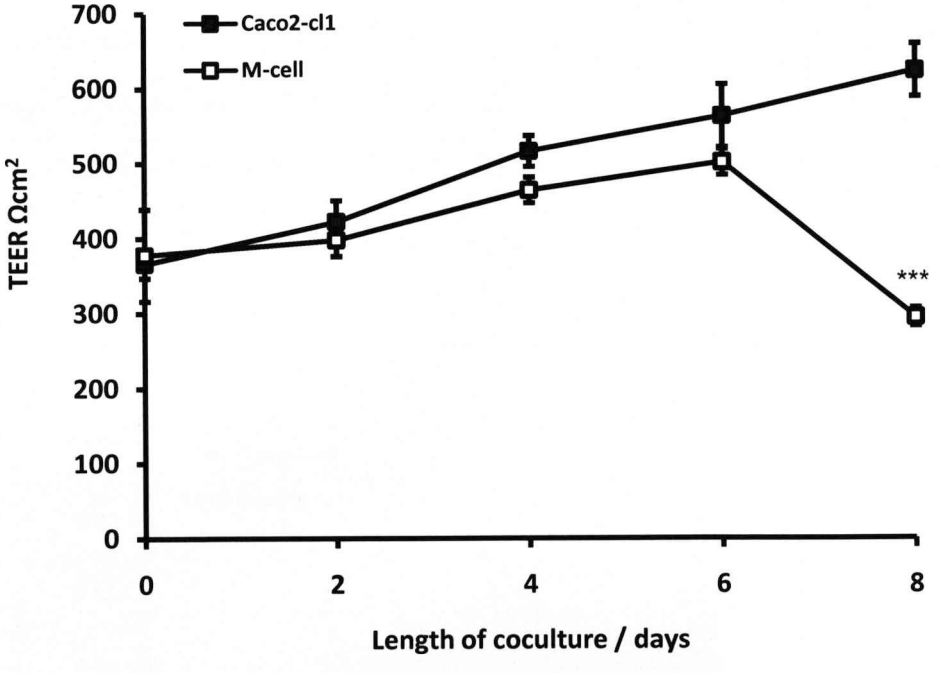


Figure 4.13 TEER changes during co-culture

The TEER of both Caco2-cl1 and M-cell modolayers increases slightly during the first 6 days of co-culture. By day 8 of co-culture, the TEER of the M-cell monolayer has fallen to less than 300 Ωcm^2, and is significantly lower than the Caco2-cl1 monolayer TEER, which remains in the region of 600 Ωcm^2 (n = 5). ***, $P < 0.001$

Following a 4 hour infection with HM605, M-cell translocation was found be significantly higher than translocation through Caco2-cl1 cells following co-culture lasting for 4, 6 and 8 days (Figure 4.14). However, it should be noted that translocation on day 8 of co-culture was not always across wells with TEER > 300 Ωcm^2. Following HM605 infection, monolayer TEER was reduced. Following infection, the TEER of both Caco2-cl1 and M-cell monolayers decreased on average by less than 10 % from the initial pre-infection TEER, when the length of co-culture was between 0 and 6 days. By the eight day of co-culture, TEER reduction following HM605 infection was greatest at 20.26 ± 4.58 % for Caco2-cl1 monolayers, and 15.79 ± 5.19 % for M-cell monolayers (Figure 4.15).

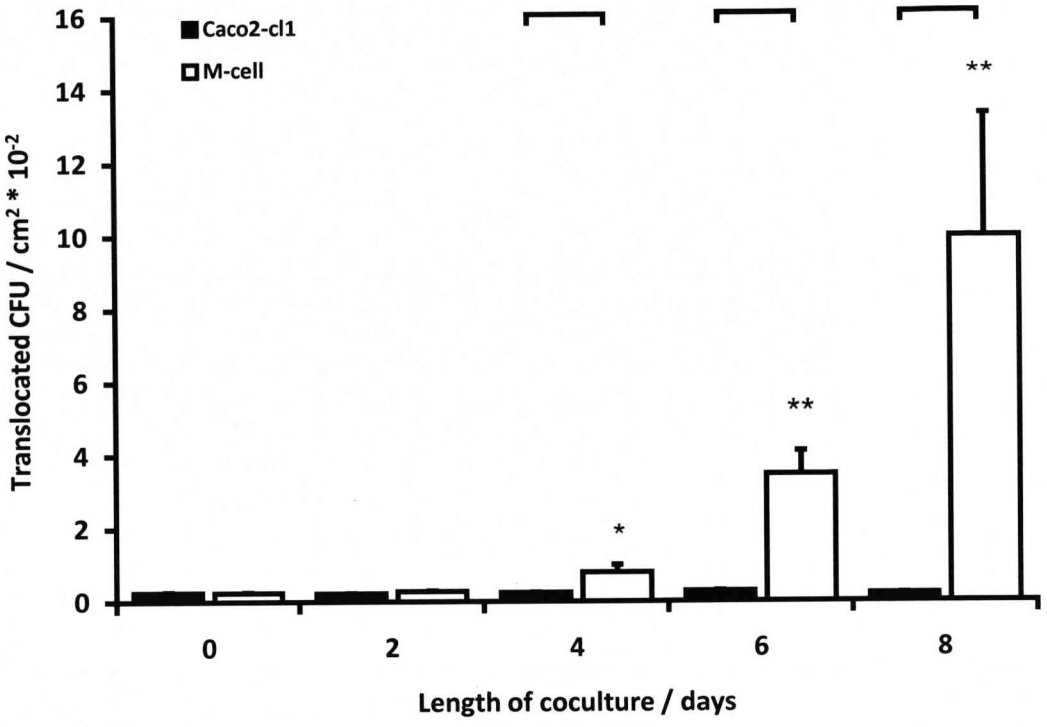


Figure 4.14 Translocation increases with length of co-culture

Translocation of Crohn's disease AIEC HM605 across Caco2-cl1 cells remains constant, whilst translocation across M-cell monolayers increases with the length of co-culture. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ (ANOVA) (n = 3)

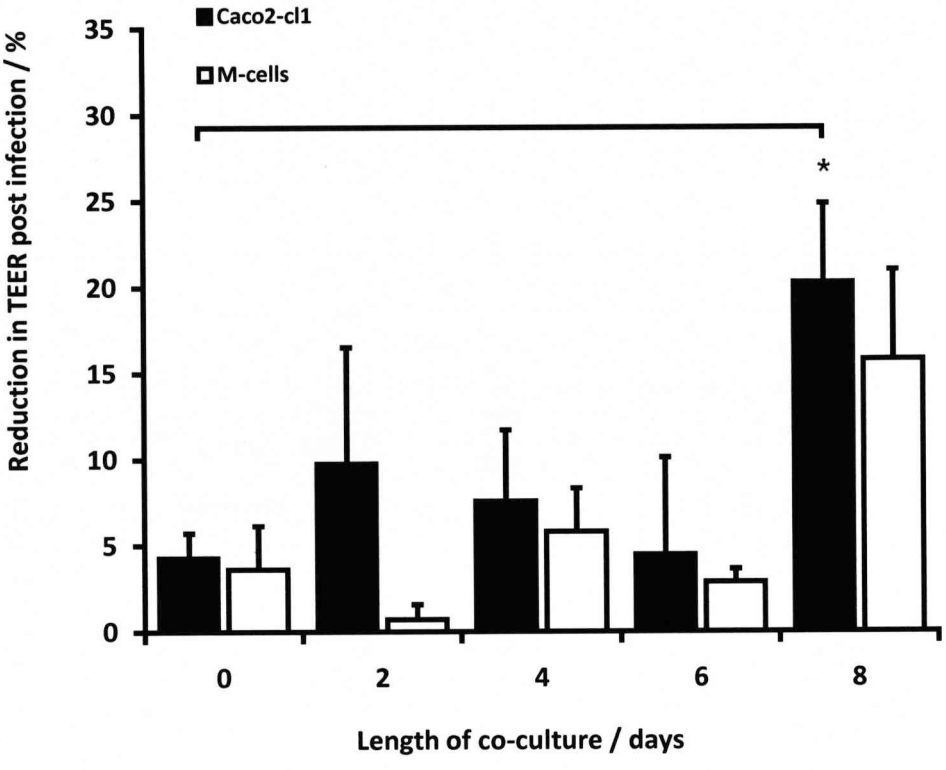


Figure 4.15 Reduction in TEER following infection

Following a 4 hour infection with HM605, the TEER of both Caco2-cl1 and M-cell monolayers decreased on average by less than 10 % from the initial pre-infection TEER, when the length of co-culture was between 0 and 6 days, whilst the TEER in monolayers cocultured for 8-days was reduced by more than 15%. *, $P < 0.05$; (ANOVA) (n = 5)

4.5.9 Raji B and Caco2-cl1 passage number alter translocation rates

It was observed following several individual assays that HM605 translocation across across M-cell monolayers was increased compared to translocation across Caco2-cl1 cells when the Raji B cells used to generate the M-cell monolayers were of a low passage number (Figure 4.16A). As the passage number of the Raji B cells increased, the ability of HM605 to translocate through the M-cell monolayer decreased, and whilst translocation was still higher than through Caco2-cl1 monolayers, the difference in translocate levels was lower, perhaps indicting that Raji B cells lose or have a decreased ability to transform Caco2-cl1 cells into M-cells as they become older. The effect of Caco2-cl1 cell passage number upon M-cell transformation appeared to have a less pronounced effect upon M-cell transformation (Figure 4.16B)

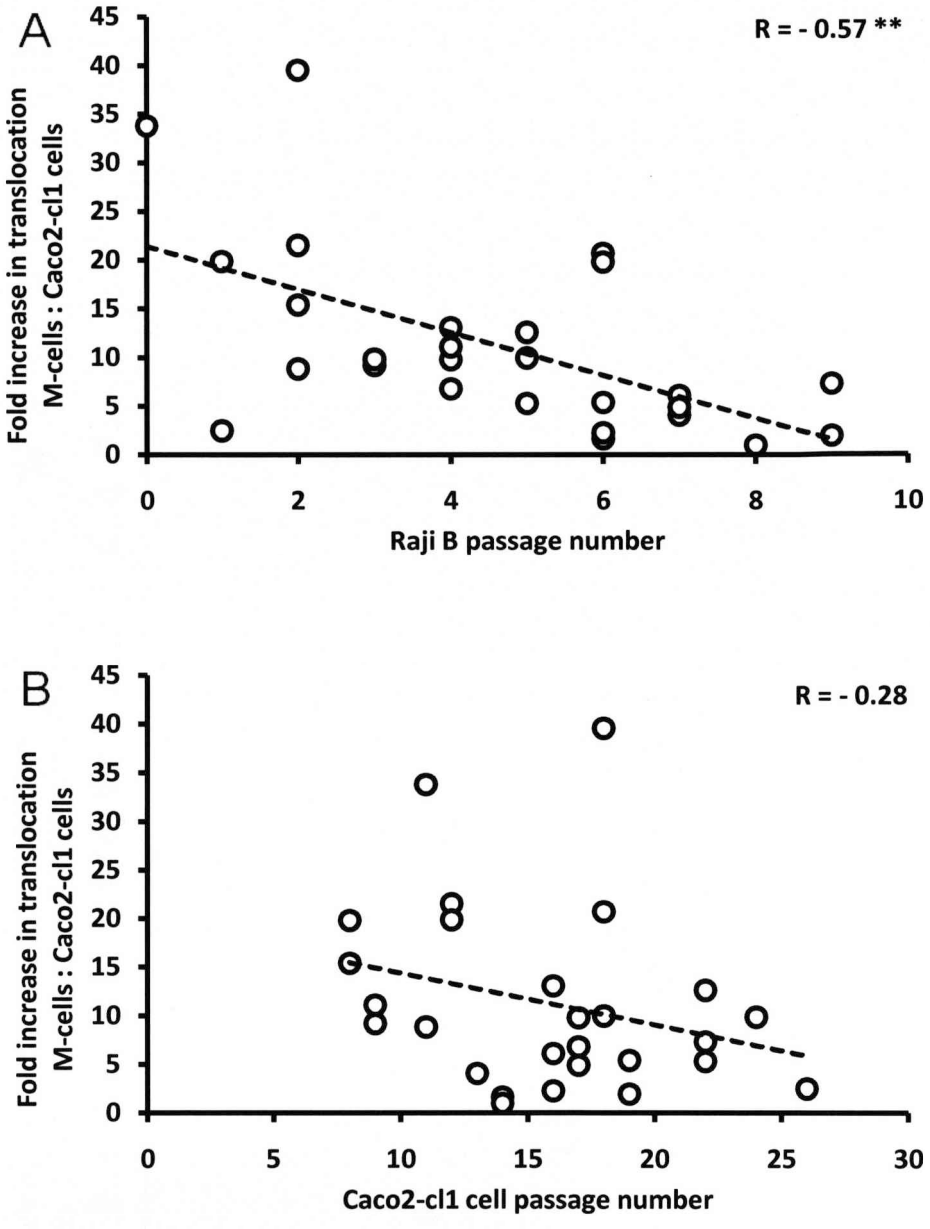


Figure 4.16 Fold increase in translocation of *E. coli* HM605 across M-cell monolayers compared with Caco2-cl1 monolayers

(A) HM605 translocation across M-cells was increased compared to translocation across Caco2-cl1 monolayer when the Raji B cells used were of a lower passage number (** $P < 0.01$, indicating significant correlation between Raji B passage number, and the fold increase in translocation of M-cells : Caco2-cl1 cells). (B). The passage number of the Caco2-cl1 cells used to generate Caco2-cl1 and M-cell monolayers appeared not to correlate with increased translocation through M-cell monolayer compared to Caco-2cl1 monolayer translocation. (n = 27, individual M-cell productions.)

4.5.10 *E. coli* HM605 growth conditions affects translocation across M-cells

It was observed that *E. coli* HM605 translocation across M-cell monolayers was dependent upon the method by which HM605 had been grown. HM605 grown overnight in LB broth translocated across M-cells much more readily than HM605 grown on LB agar, or LB agar followed by 48 h suspension in PBS, during an 8 hour infection period (Figure 4.17A). There appeared to be no difference between translocation of HM605 grown on LB agar, or LB agar followed by 48 h suspension in PBS across M-cell monolayers over the same 8 hour infection period (Figure 4.17B). Following a 24 hour infection, there was no difference between the translocation across M-cells and the growth condition of HM605 (Figure 4.17C). There was however a significant reduction in M-cell monolayer TEER following infection with LB broth grown HM605 at all time points investigated (Figure 4.18A), and following 24 hours of infection, a significant reduction in M-cell monolayer TEER for all HM605 growth conditions (Figure 4.18B), indicating monolayer disruption may be a contributing factor in increased translocation rates. LB agar grown HM605 saw least disruption to TEER, indicating it is a suitable growth condition for future studies.

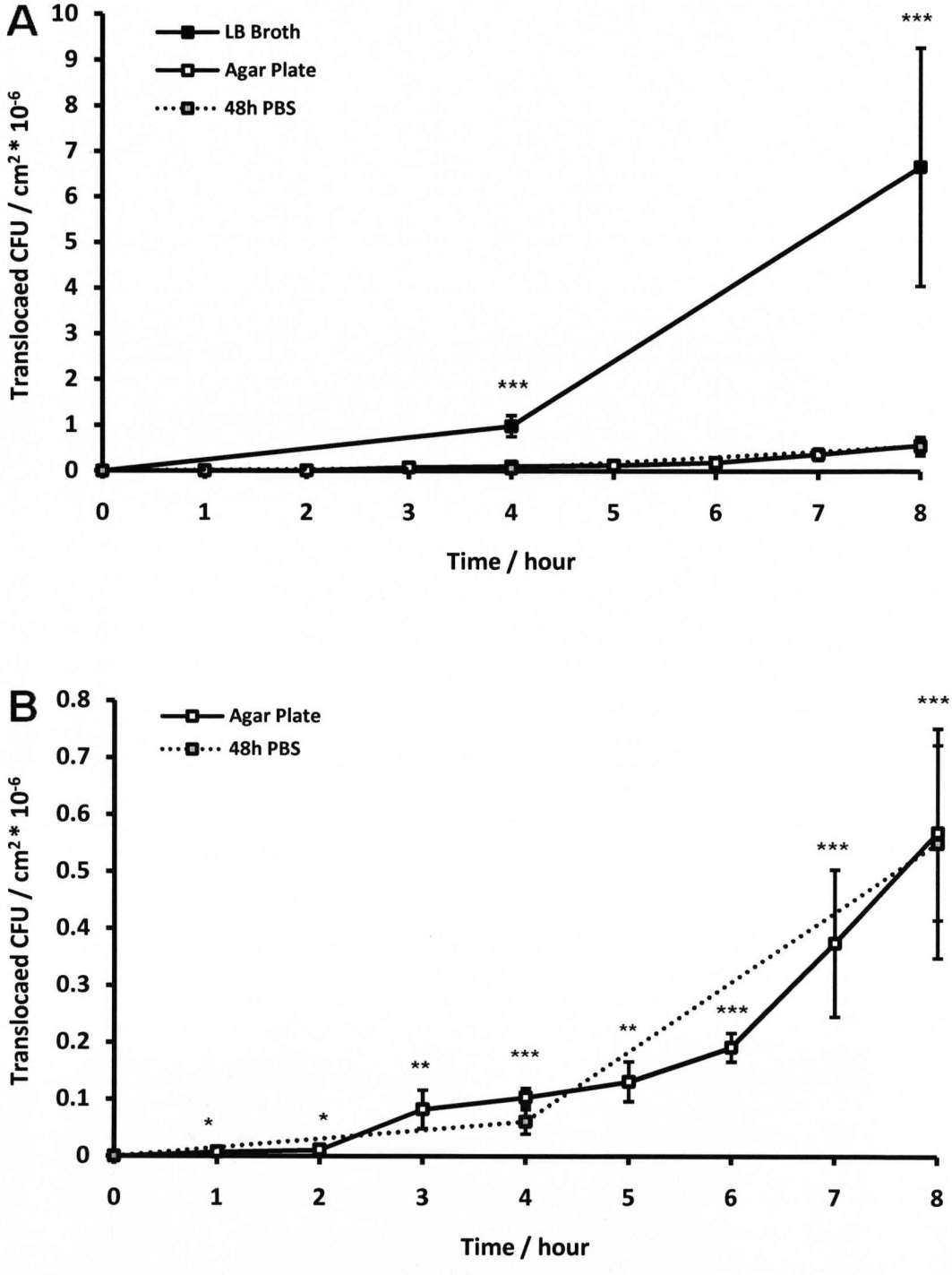


Figure 4.17 Method of growth of HM605 alters translocation across M-cell monolayers

Legend overleaf

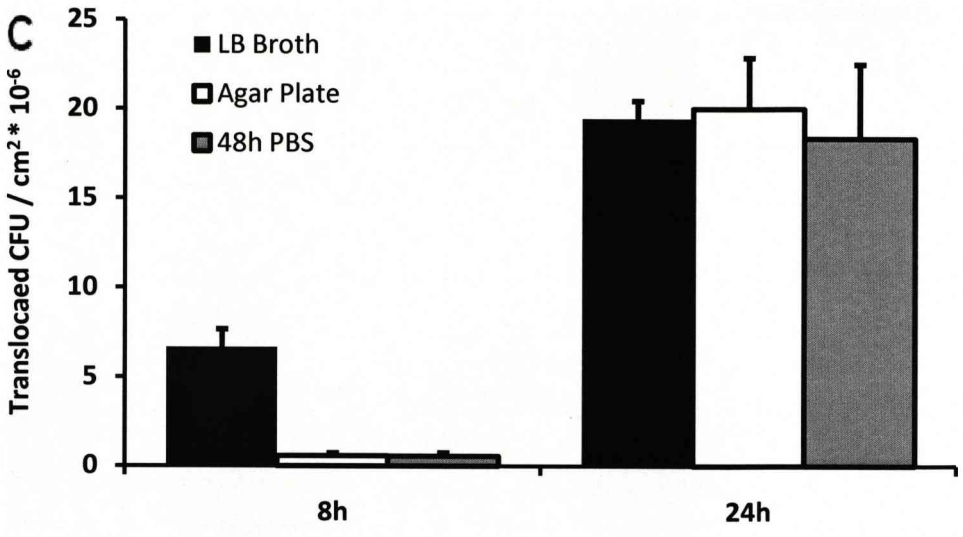


Figure 4.17 (continued) Method of growth of HM605 alters translocation across M-cell monolayers

(A) HM605 grown in LB broth overnight translocates across M-cells to a greater extent than HM605 grown on solid LB agar overnight, or grown on LB agar followed by 48 hour suspension in sterile PBS. Translocation at the time points investigated, 4 h and 8 h, is significantly higher than translocation at 0 h. (B). Data from panel A redrawn for greater clarity. There appears to be no difference in translocation between HM605 grown on solid LB agar which has been left for 48 h in PBS, and those which have not. Translocation is significantly higher at all time points investigated than translocation at 0 h. (C) Following 24 hours of infection, there was no difference between the levels of translocation across M-cell monolayers and the method by which HM605 was grown. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ (ANOVA) (n = 3)

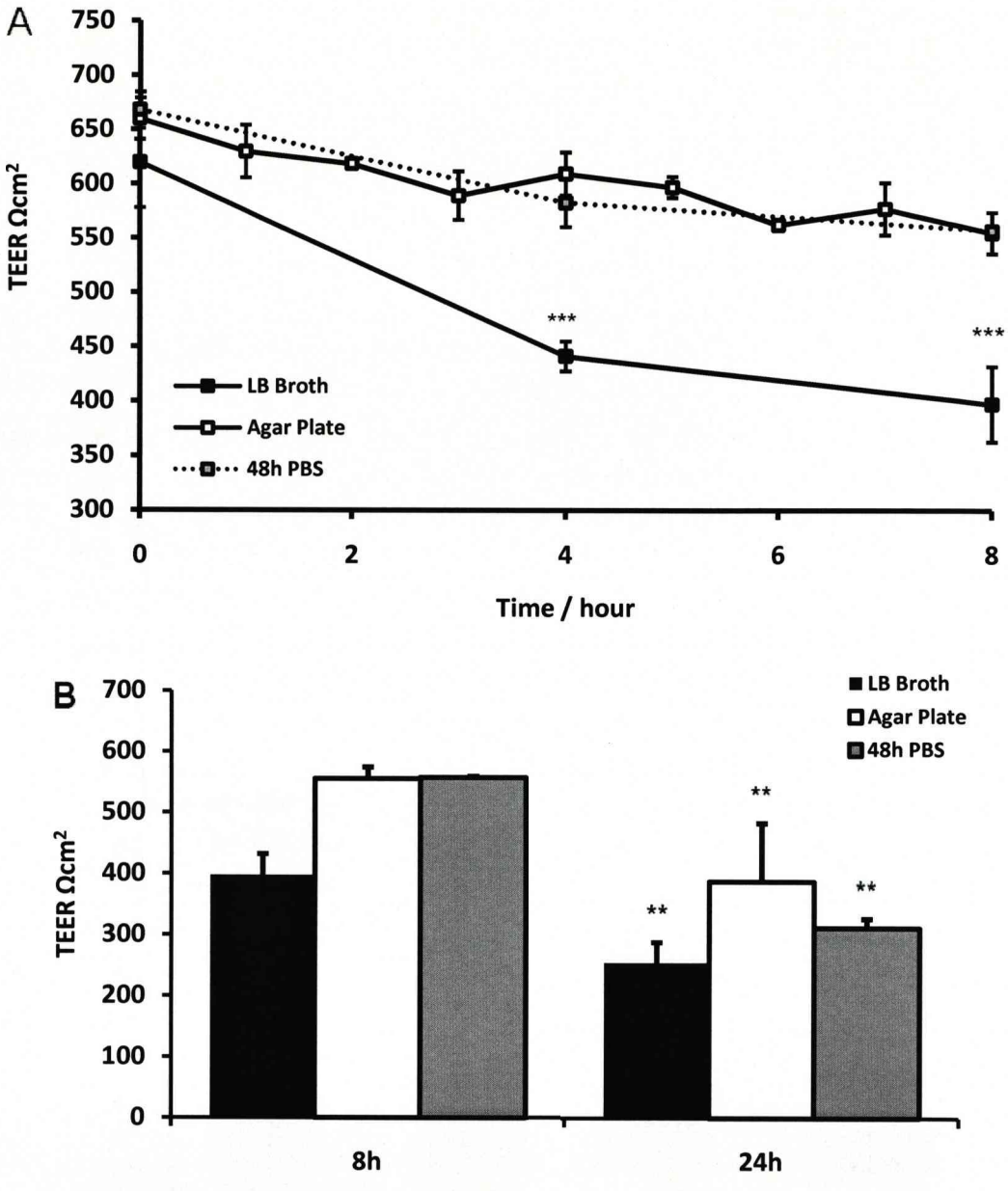


Figure 4.18 TEER alterations in response to differently grown HM605

(A) HM605 grown in LB broth led to a significant reduction in TEER following 4 hours and 8 hours of infection. (B) Following 24 hours of infection, there was a significant reduction in M-cell monolayer TEER for all HM605 growth conditions. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ (ANOVA) (n = 3)

4.5.11 HM605 translocation across M-cell monolayers occurs sooner than translocation across Caco2-cl1 monolayers

Translocation of HM605 across Caco2-cl1 monolayers was detectable 30 minutes post infection, at low levels. Translocation across M-cell monolayers was detectable within 15 minutes post-infection (Figure 4.19A). Monolayer TEER was not affected by infection (Figure 4.19B).

4.5.12 HM605 translocation is not dependent upon TEER

Translocation of HM605 across both Caco2-cl1 and M-cell monolayers following a 4 hour infection was found to be independent of monolayer TEER (Figure 4.20), providing TEER > 300 Ωcm^2.

4.5.13 HM605 was detected within M-cell monolayers

Translocation of HM605 through rather than between cells of the M-cell monolayer was confirmed by TEM. HM605 was observed within M-cell monolayer cells, but could not be detected either within Caco2-cl1 monolayers, or between cells of the monolayer (Figure 4.21).

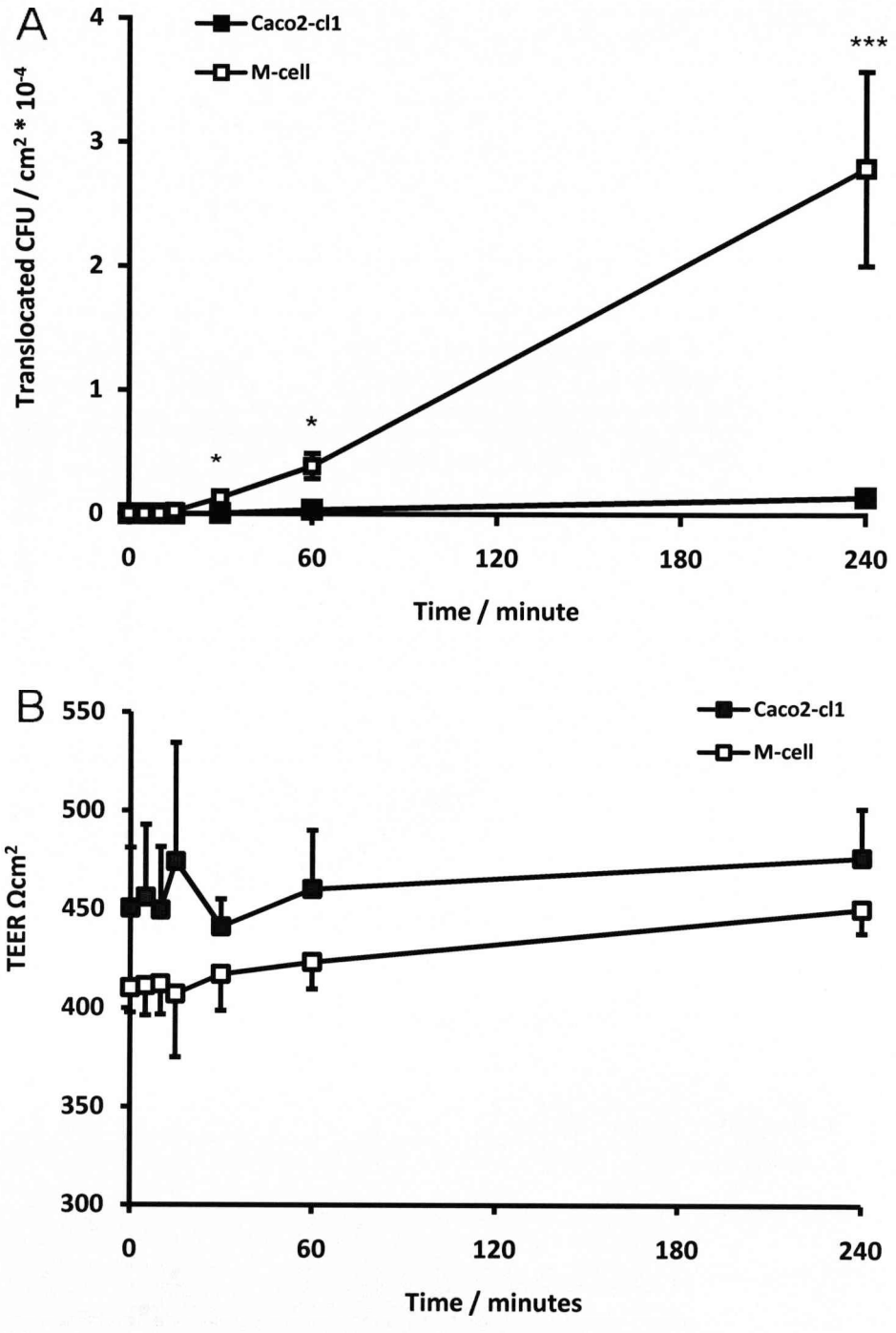


Figure 4.19 Translocation occurs sooner across M-cell monolayers than across Caco2-cl1 monolayers

(A) Translocation across M-cell monolayers was first quantifiable within 15 minutes post infection, whilst translocation across Caco2-cl1 monolayers could not be detected until at least 30 minutes post infection. (B) In this assay, TEER remained above 300 Ωcm^2 throughout the course of infection. Monolayer TEER was slightly elevated following 4 hour infection with HM605. ($n \geq 5$). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ (ANOVA) ($n = 3$)

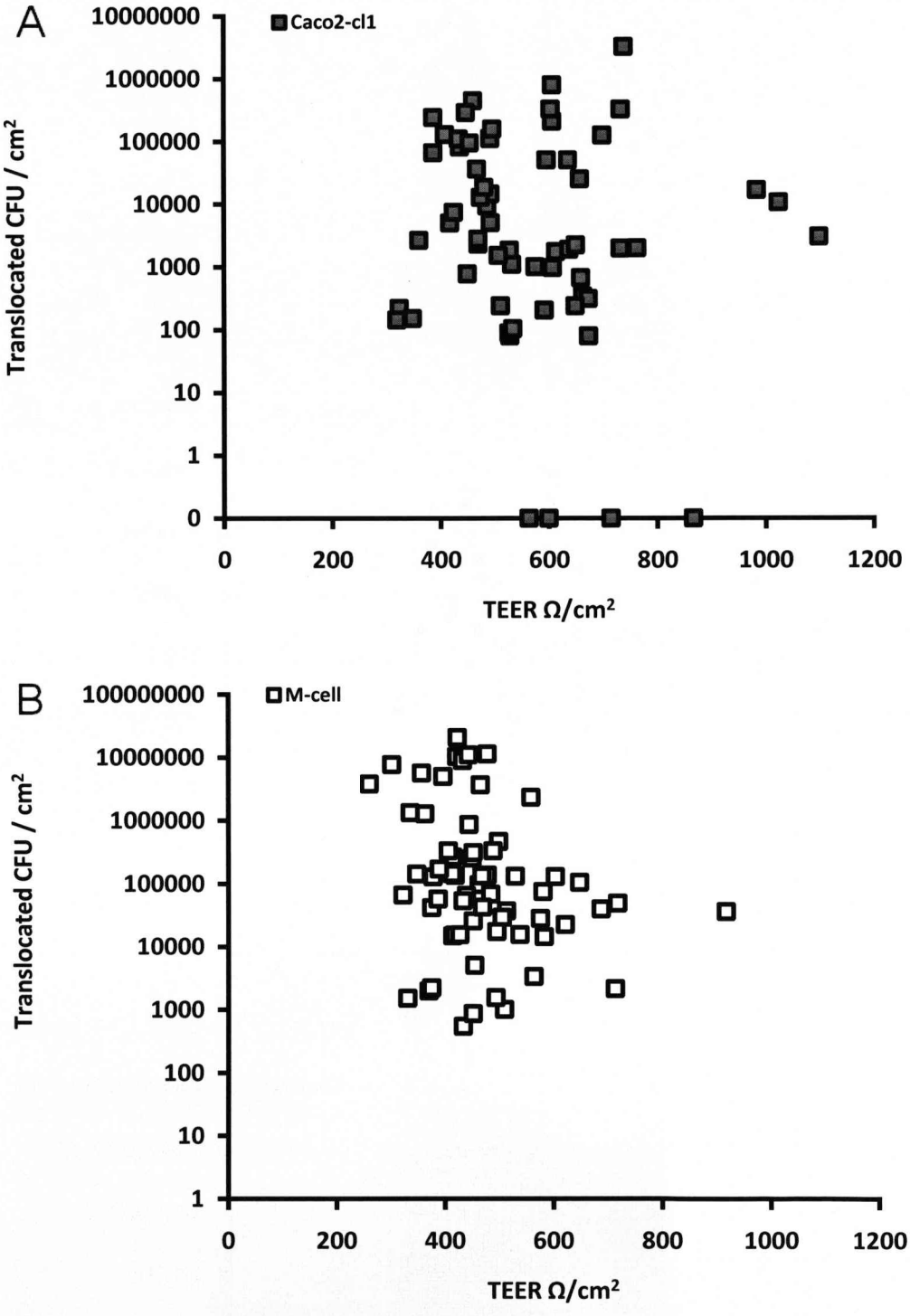


Figure 4.20 Translocation is independent of TEER

(A) Caco2-cl1 translocation of Crohn's disease AIEC HM605 was independent of TEER, when TEER ≥ 300 Ωcm^2. (n > 60). (B). M-cell translocation of Crohn's disease AIEC HM605 was independent of TEER, when TEER ≥ 300 Ωcm^2 (n > 60).

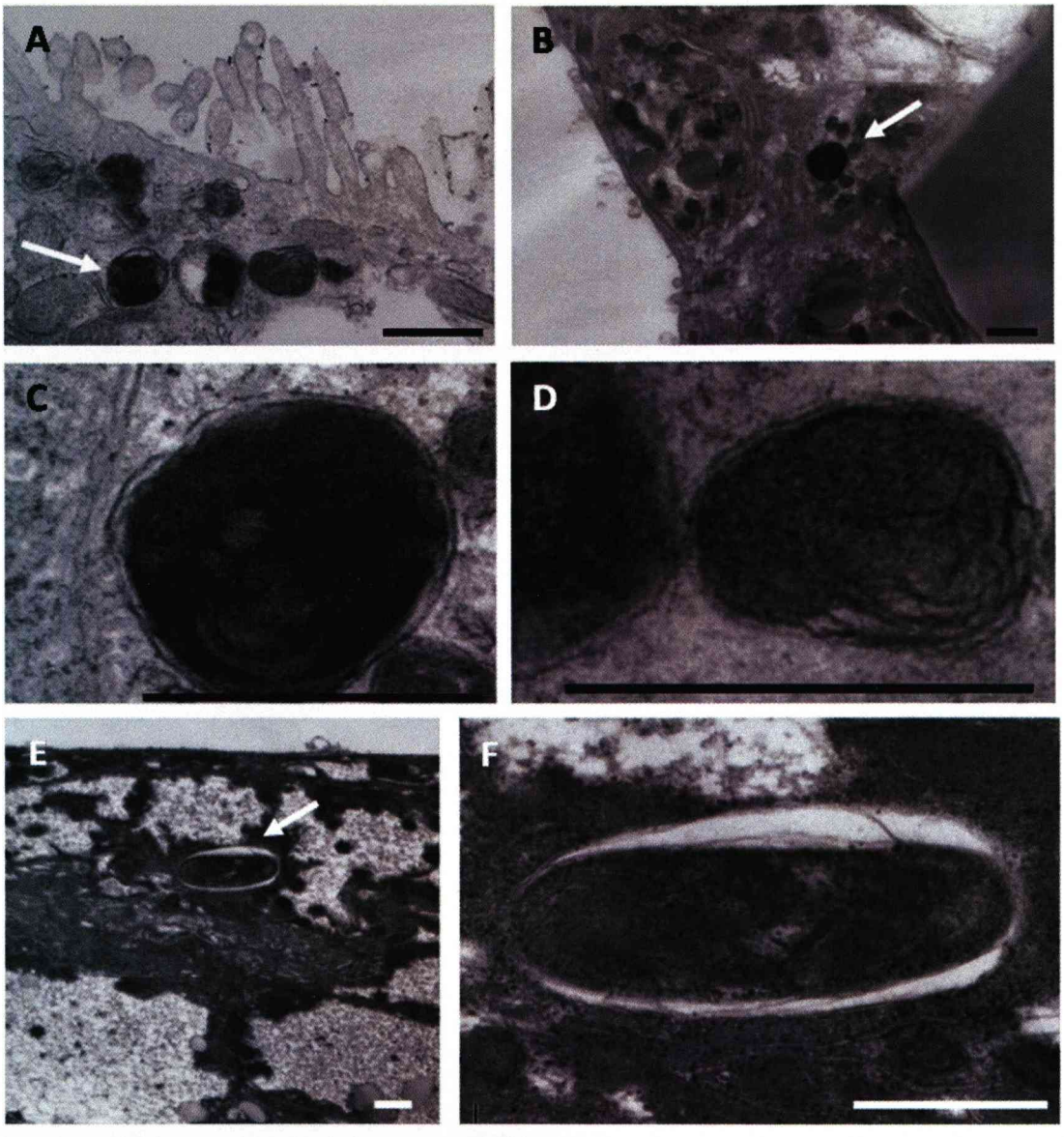


Figure 4.21 HM605 within M-cell monolayers

(A – E) TEM analysis of *E. coli* HM605 within M-cell monolayers, indicated by white arrows in A, B and E. (F) An enlargement of image E, showing HM605 more clearly. *E. coli* HM605 was not observed within Caco2-cl1 monolayers. Bar = 0.5µm

4.5.14 Translocation of Crohn's disease *E. coli* is increased across M-cell monolayers

Eight Crohn's disease AIEC were tested for their ability to translocate through M-cell monolayers. Each translocated through M-cell monolayers much more so than through Caco2-cl1 monolayers. Four of these isolates, HM580, HM605, HM615 and LF82 translocated through M-cells more significantly than *E. coli* K12 ($P < 0.05$), whilst four strains did not (HM95, HM154, HM413, HM419) (Figure 14.22). Interestingly, those isolates, with the exception of LF82, which did translocate through M-cells more than *E. coli* K12 were those isolates which has previously been shown to agglutinate red blood cells, whilst those that did not translocate through M-cells more than *E. coli* K12 were also found not to agglutinate red blood cells.

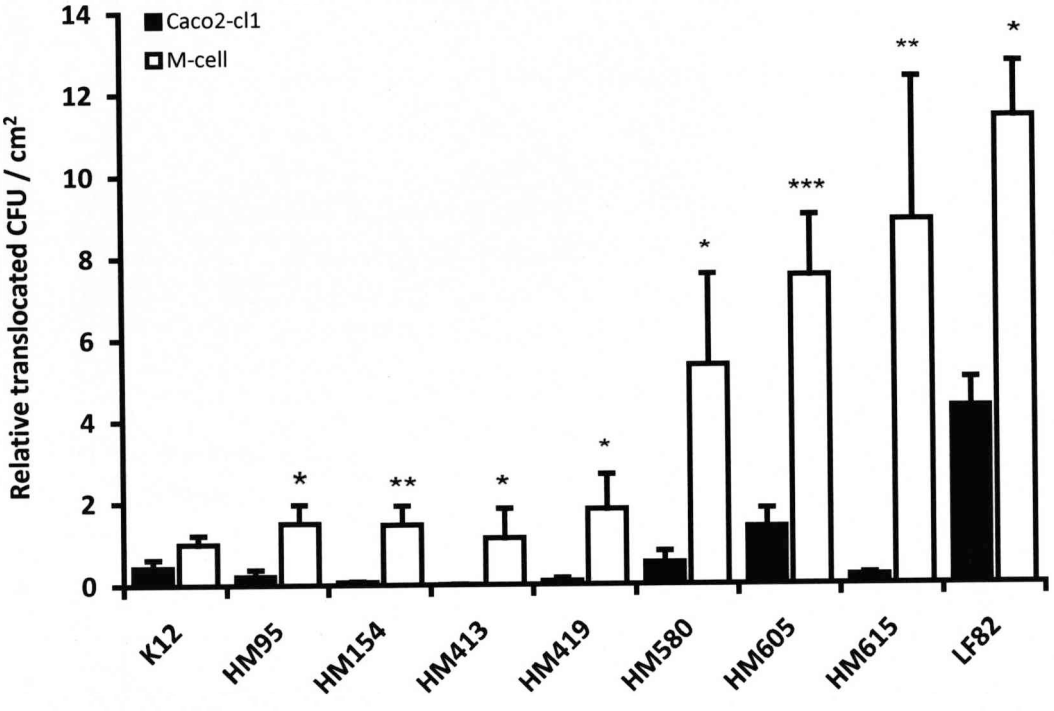


Figure 14.22 Translocation of Crohn's disease AIEC

Crohn's disease *E. coli* isolates are translocated through M-cell monolayers more readily than through Caco2-cl1 monolayers (n≥3). *, $P < 0.5$; **, $P < 0.01$; and ***, $P < 0.001$ (ANOVA).

4.5.15 Translocation of control patient *E. coli* is increased across M-cell monolayers

A panel of five control patient *E. coli* isolates (irritable bowel syndrome / sporadic polyposis) were tested for their ability to translocate through Caco2-cl1 and M-cell monolayers. Two of the strains investigated, HM488 and HM456 were found to significantly translocate through M-cell monolayers much more readily than through Caco2-cl1 monolayers, and more so than control *E. coli* K12 (Figure 4.23). TEERS were in excess of 300 Ωcm^2 for each *E. coli* isolate investigated throughout the course of the 4 hour infection, indicating that monolayer integrity was maintained throughout infection.

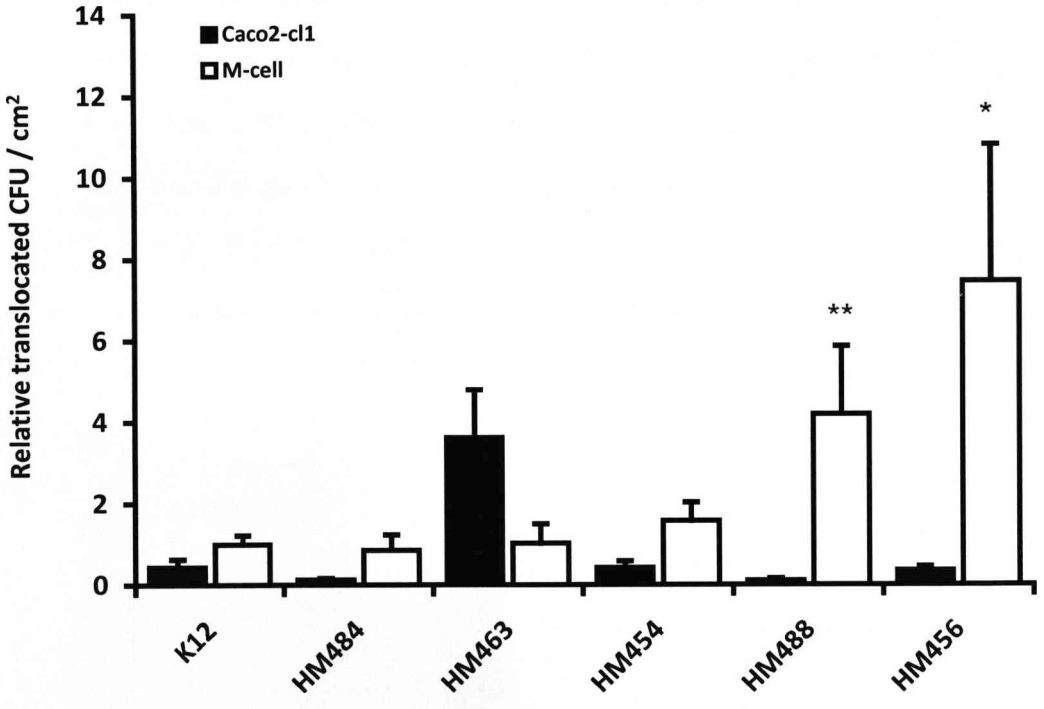


Figure 4.23 Translocation of control patient AIEC

Control patient (irritable bowel syndrome / sporadic polyposis) mucosal adherent and invasive *E. coli* isolates are translocated through M-cell monolayers more readily that through Caco2-cl1 monolayers, with the exception of *E. coli* HM463 ($n \geq 3$), following a 4 hour infection. *, $P < 0.5$; and **, $P < 0.01$ (ANOVA).

4.6 SUMMARY OF RESULTS

4.6.1 Features of *in vivo* M-cells validated in an *in vitro* M-cell model

1. *In vitro* derived M-cells have a varied expression of apical microvilli, unlike Caco2-cl1 cells which have a regular expression of apical microvilli.

2. *In vitro* derived M-cells have increased binding of *Aleuria aurantia* lectin, and decreased expression of total alkaline phosphatase protein.

3. M-cell monolayers translocate *Salmonella* Typhimurium LT2 and *Shigella sonnei* 80-fold and 8-fold more than Caco2-cl1 monolayers respectively, independently of monolayer TEER.

4.6.2 Characterisation of translocation of *E. coli* HM605 across M-cells and Caco2-cl1 cells

1. The length of Caco2-cl1 monolayer co-culture with Raji B cells affects translocation of *E. coli* HM605, and the resultant TEER of the monolayer, both prior to, and during infection. The optimal length of Caco2-cl1 co-culture with Raji B cells is between 4 – 6 days.

2. Following a 30 minute infection, M-cell monolayers with TEER > 200 Ωcm^2 were found only to translocate very low numbers of HM605 (less than 0.1 % of the initial inoculum), whilst monolayers with TEER < 200 Ωcm^2 saw much higher numbers of HM605 passing through the monolayer (1 – 10 %).

3. Raji B cells with a lower passage number co-cultured with Caco2-cl1 monolayers generally led to the formation of M-cell monolayers which had a higher rate of translocation of *E. coli* HM605 when compared to Caco2-cl1 monolayer translocation.

4. The method by which *E. coli* HM605 is grown appears to affect both translocation rates and monolayer TEER during infection. The optimal growth condition appears to be overnight growth on LB agar.

5. HM605 translocation across M-cell monolayers (when TEER > 300 Ωcm^2) is just detectable at 15 minutes, and then increases steadily over time, without significant disruption to the monolayer integrity. Translocation across Caco2-cl1 cells was just detectable after 30 minutes, and again, translocation increased over time. Translocation was not dependent upon TEER when TEER > 300 Ωcm^2.

6. HM605 was detected within cells of M-cell monolayers by TEM, but not in Caco2-cl1 monolayers, or between cells.

4.6.3 Translocation of *E. coli* across M-cells

1. Translocation of *E. coli* K12, *E. coli* XL1-Blue and probiotic *E. coli* Nissle 1917 across M-cell monolayers does not appear to be significantly different to translocation across Caco2-cl1 monolayers.

2. All Crohn's disease AIEC investigated translocated through M-cell monolayers to a greater extent than through Caco2-cl1 monolayers.

3. Two of the five control subject AIEC *E. coli* translocated through M-cell monolayers to a greater extent than through Caco2-cl1 monolayers

4.7 DISCUSSION

The generation of *in vitro* M-cells was confirmed structurally by the use of transmission electron microscopy. Caco2-cl1 monolayers which had been co-cultured with Raji B cells to form M-cells were found to have a distinct lack of microvilli when compared to Caco2-cl1 monolayers alone, indicating the lack of a brush boarder on M-cells compared to Caco2-cl1 cells. This type of structural alteration has previously been reported by Kerneis *et al.* [Kerneis *et al.* 1997] and Gullberg *et al.* [Gullberg *et al.* 2000].

Functionally, these *in vitro* derived M-cells were able to translocate *Salmonella* Typhimurium LT2 and *Shigella sonnei* from the apical to basolateral aspect of the cell monolayer; these findings have also been observed *in vivo* [Jepson *et al.* 2001].

Total alkaline phosphatase expression was also reduced in this *in vitro* M-cell model, by 21.6 ± 4.1 % compared to Caco2-cl1 cell monolayers. This is consistent with the work of Gullberg *et al.* who also reported a decrease in apical alkaline phosphatase activity of ~ 35 % [Gullberg *et al.* 2000]. It is also consistent with the reported downregulation of alkaline phosphatase activity of M-cells seen *in vivo* [Owen *et al.* 1983; Farstad *et al.* 2002].

The increased binding of *Aleuria aurantia* lectin (AAL) to M-cell monolayers compared with Caco2-cl1 monolayers is interesting as it supports the hypothesis that AAL could be used to target M-cells *in vivo* [Roth-Walter *et al.* 2004; Roth-Walter *et al.* 2005].

The use of Crohn's disease *E. coli* to characterise translocation across M-cell monolayers revealed some interesting findings. Translocation occurred within 15 minutes across M-cell monolayers but was only detected at 30 minutes for Caco2-cl1 monolayers

This work demonstrates that the *in vitro* M-cell model can be used to establish bacterial translocation of Crohn's disease and control patient *E. coli* across cell monolayers *in vitro*. Translocation of all Crohn's disease *E. coli* isolates investigated was consistently higher across M-cells than Caco2-cl1 cells, whilst 5 / 6 control subject *E. coli* were translocated across M-cells to a greater extent than across Caco2-cl1 cells. Translocation was also noticed to occur sooner across M-cells than Caco2-cl1 cells; translocation was first quantifiable 15 minutes post infection for M-cells, but only at 30 minutes, and to a much lower extent, in Caco2-cl1 cells.

The cause for this increased translocation across M-cells is unclear; the lack of microvilli in M-cells would certainly allow *E. coli* interaction with the cell surface without the physical barrier that the microvilli normally provide. Increased access of *E. coli* to the M-cell surface would perhaps in turn lead to an increase in bacterial uptake and translocation. It is also plausible that the *E. coli* themselves are somehow targeted to the M-cell rather than Caco2-cl1 cells, through expression of surface fimbri or other adhesion molecules not yet fully understood.

It is interesting that the three Crohn's disease *E. coli* which translocate across M-cells to the greatest extent (HM580, HM605, HM615) are also all able to haemagglutinate red blood cells (Appendix 2) [Martin 2004; Martin *et al.* 2004]. The non-haemagglutinating Crohn's disease *E. coli* (HM95, HM154, HM413) all translocate across M-cells more readily than across Caco2-cl1 cells, but the absolute level of translocation across M-cells is lower than that of the three haemaggutinating strains. HM419, a Crohn's disease haemagglutinating strain was the exception – it's translocation across M-cells was similar to that of the non-haemaglutinating strains. Interestingly, the classical AIEC LF82, a non-haemagglutinating strain, was translocated across M-cells to a greater extent than HM95, HM154 and HM413, and at a comparible level to the haemagglutinating strains HM580, HM615 and HM615.

It is also interesting that the Crohn's disease *E. coli* belonging to the B2 and D phylotype (HM580, HM605, HM615, LF82) were translocated through M-cells to a greater extent than the Crohn's disease *E. coli* belonging to the A and B1 phylotype (HM95, HM154). (The B2 and D phylotype is associated with uropathic, avian and extraintestinal virulent *E. coli* strains (Uropathogenic

(UPEC), avian pathogenic (APEC) and Extraintestinal pathogenic (ExPEC)) [Kotlowski *et al.* 2007].)

Two control patient AIEC were translocated across M-cells more significantly than across Caco2-cl1 cells, both of these strains (HM488 and HM456) belonged to the B2 phylotype, but one (HM456) was a haemagglutinating strain, whilst HM488 was a non-haemagglutinater.

Whilst these observations are based on only small numbers of *E. coli* strains, it does indicate that translocation of Crohn's disease *E. coli* isolates across M-cells might be subject to the unique properties of each individual *E. coli*. Additionally, the presence of increased translocation across M-cells does support the hypothesis that *E. coli* transport at this site *in vivo* might be involved in Crohn's disease pathogenesis. Indeed, *ex vivo* work conducted with macroscopically normal Crohn's disease FAE biopsies show increased numbers of adherent bacteria compared to control patient on the FAE [Keita *et al.* 2008]. It is plausible that increased bacterial load at M-cells could be important in the development of Crohn's disease.

Under normal circumstances, microbota passing through the intestinal barrier would be expected to be cleared by a robust phagocytic system. In Crohn's disease however, there is evidence for a defective innate immune system as documented by evidence of impaired neutrophil recruitment [Marks *et al.* 2006]. This leads to the hypothesis that AIEC gain entry via the M-cells, are inadequately cleared by neutrophils, and become ingested by macrophages where they replicate within endophagolysosomes and stimulate granuloma formation. The replication of the typical ileal AIEC isolate LF82 has already been observed with macrophages [Glasser *et al.* 2001].

Despite the findings presented here that Crohn's disease *E. coli* isolates are translocated across *in vitro* M-cells, it does not prove categorically that these *E. coli* have a causative role in the pathogenesis of Crohn's disease. Whilst the correlation between Crohn's disease *E. coli* translocation across M-cells and the location of the initial lesions observed in Crohn's disease would imply that *E. coli* might be a cause of the initial lesion, it is also perfectly possible that *E. coli* translocation at M-cells is a perfectly normal intestinal occurrence, and not related to the initiation of the disease itself. It is estimated that M-cells account for only 0.01 % of the total intestinal epithelium [Jepson *et al.* 2001]. Bacterial invasion at the site of M-cells is thus restricted to a relatively small proportion of the total cells within the intestinal epithelium, implying that they're impact upon Crohn's disease may be small.

Chapter 5

Impact of Dietary Factors upon Bacterial Translocation across Caco2-cl1 and M-cell monolayers

5.1 HYPOTHESIS

Soluble plant fibres such as plantain, leek, apple and broccoli NSP will inhibit bacterial translocation across *in vitro* derived M-cells. In contrast, food emulsifiers such as polysorbate-60 and polysorbate-80 will lead to increased bacterial translocation across *in vitro* derived M-cells.

5.2 AIMS

1. To determine the effect of plant NSPs upon bacterial translocation
2. To determine the effect of common food emulsifiers upon bacterial translocation

5.3 INTRODUCTION

Crohn's disease is accepted as being due to a combination of genetic and environmental factors. The genetic factors include mutations that affect handling of bacteria by the innate immune system [Hugot *et al.* 2001; Ogura *et al.* 2001; 2007; Hampe *et al.* 2007; Levine *et al.* 2007; Parkes *et al.* 2007; Zhernakova *et al.* 2008], but the environmental factors, aside from smoking [Calkins 1989; Cottone *et al.* 1994], are poorly understood. There are marked geographical variations in Crohn's disease incidence, with lower prevalence in underdeveloped countries, and a rapid recent rise in Japan that has paralleled the introduction of a 'Western diet' [Sakamoto *et al.* 2005]. It is therefore a plausible hypothesis that dietary factors may modulate the relationship between the host epithelia and the intestinal microbiota.

The adhesion and invasion of AIEC to intestinal epithelial cells is inhibited by soluble plantain fibre [Martin *et al.* 2004]. It is interesting that in parts of the World such as Africa, India and Central America where plantain form an important part of the staple diet, have low rates for inflammatory and malignant colonic disease. It is theoretically possible that other soluble plant based fibres may have similar effects upon bacterial invasion and adhesion.

The lactulose/mannitol test measures the ratio of urinary excreted lactulose:mannitol following oral ingestion [Söderholm *et al.* 1999], it demonstrates that intestinal permeability is increased in both Crohn's disease patients [Benjamin *et al.* 2008], and in their unaffected relatives [Katz *et al.* 1989; Söderholm *et al.* 1999; D'Inca *et al.* 2006]. It is possible that environmental factors may lead to this increased intestinal permeability [Breslin *et al.* 2001]. The increase in Crohn's disease incidence seen in Japan correlates with increased fat intake there – and so increased consumption of emulsifiers within food. It is plausible that emulsifiers within the diet may lead to increased intestinal permeability.

5.4 METHODS

Control Caco2-cl1 cell monolayers and M-cell monolayers were generated as described previously. Detailed descriptions for each assay can be found within Chapter 2. Briefly, the apical surface of cell monolayers were pre-treated with plant NSP (0.05 – 50 mg / mL) or food emulsifiers (in the range 0.0001 – 0.1 % v/v), prior to bacterial infection. Post infection, translocated bacteria were quantified from the basolateral aspect of the monolayer. Data

are presented as translocated CFU per cm^2 monolayer; where this is not possible, data are presented as relative translocated CFU, where the translocated CFU are expressed relative to either translocation of *E. coli* K12 (set at 1), or translocation in the absence of plant fibres or emulsifiers (also set at 1).

5.5 RESULTS

5.5.1 Plantain NSP inhibits HM605 translocation across M-cell monolayers

In the absence of plantain NSP, translocation of HM605 was approximately 20 fold higher through M-cell monolayers compared to Caco2-cl1 monolayers, indicating successful generation of M-cell monolayers. Following plantain treatment at 5 and 50 mg / mL, translocation of HM605 across M-cells was inhibited (percentage inhibition in translocation, 5 mg / mL; 82.6 ± 7.2 %; 50 mg / ml, 86.2 ± 11.6 %) (Figure 5.1A). Translocation of HM605 across Caco2-cl1 monolayers was not significantly inhibited by the presence of plantain at the investigated doses of 0.05 – 50 mg / mL plantain (Figure 5.1B). Plantain treatment at all concentrations did not affect monolayer TEER (Figure 5.2).

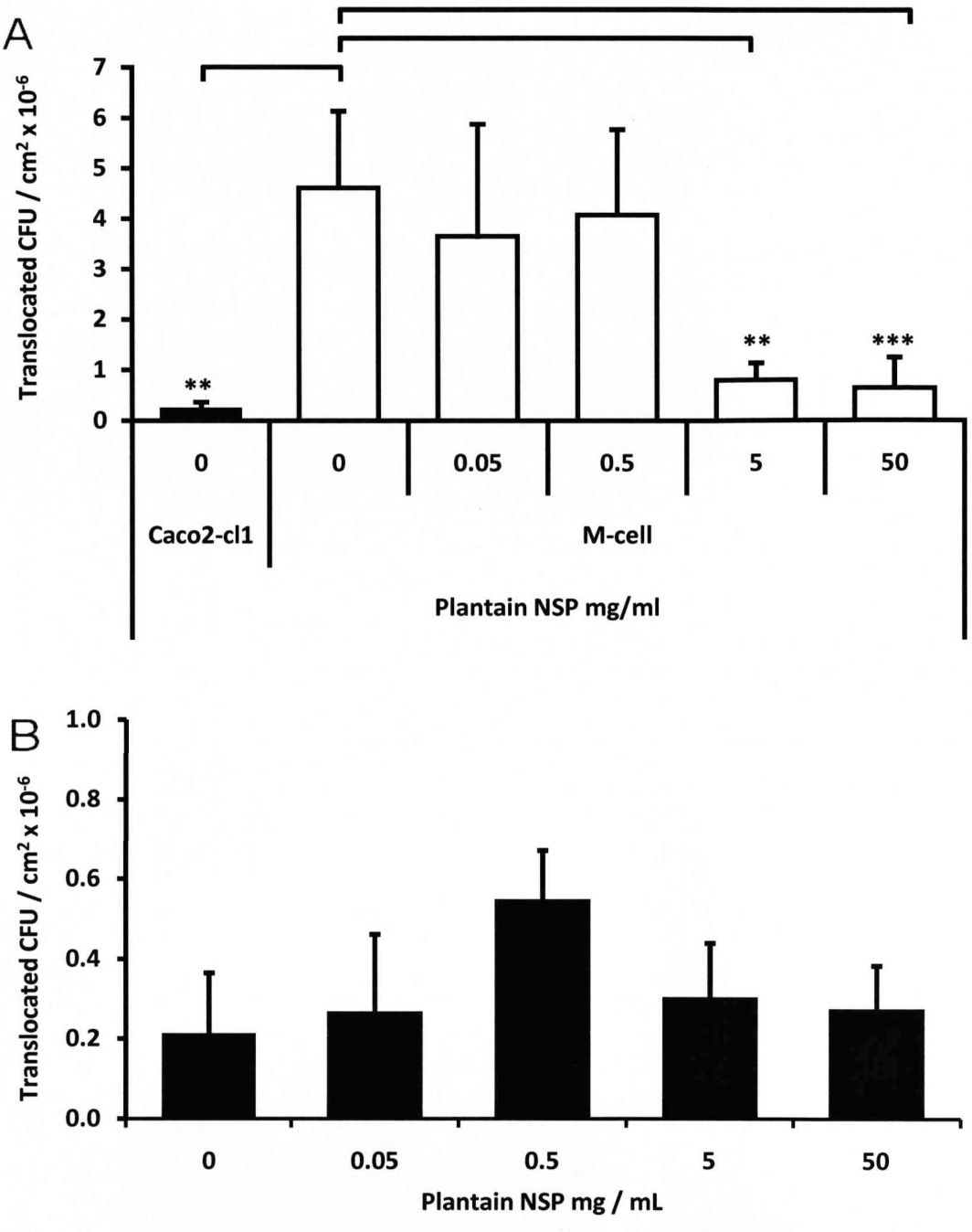


Figure 5.1 Plantain NSP affects *E. coli* HM605 translocation across M-cells, but not across Caco2-cl1 cells

(A) *E. coli* HM605 translocation through M-cells is inhibited by the presence of plantain NSP at the investigated concentrations of 5 mg / mL and 50 mg / mL. (ANOVA) (n = 6) (B) Plantain NSP does not affect HM605 translocation across Caco2-cl1 monolayers. **, $P < 0.01$; and ***, $P < 0.001$ (ANOVA) (n = 3)

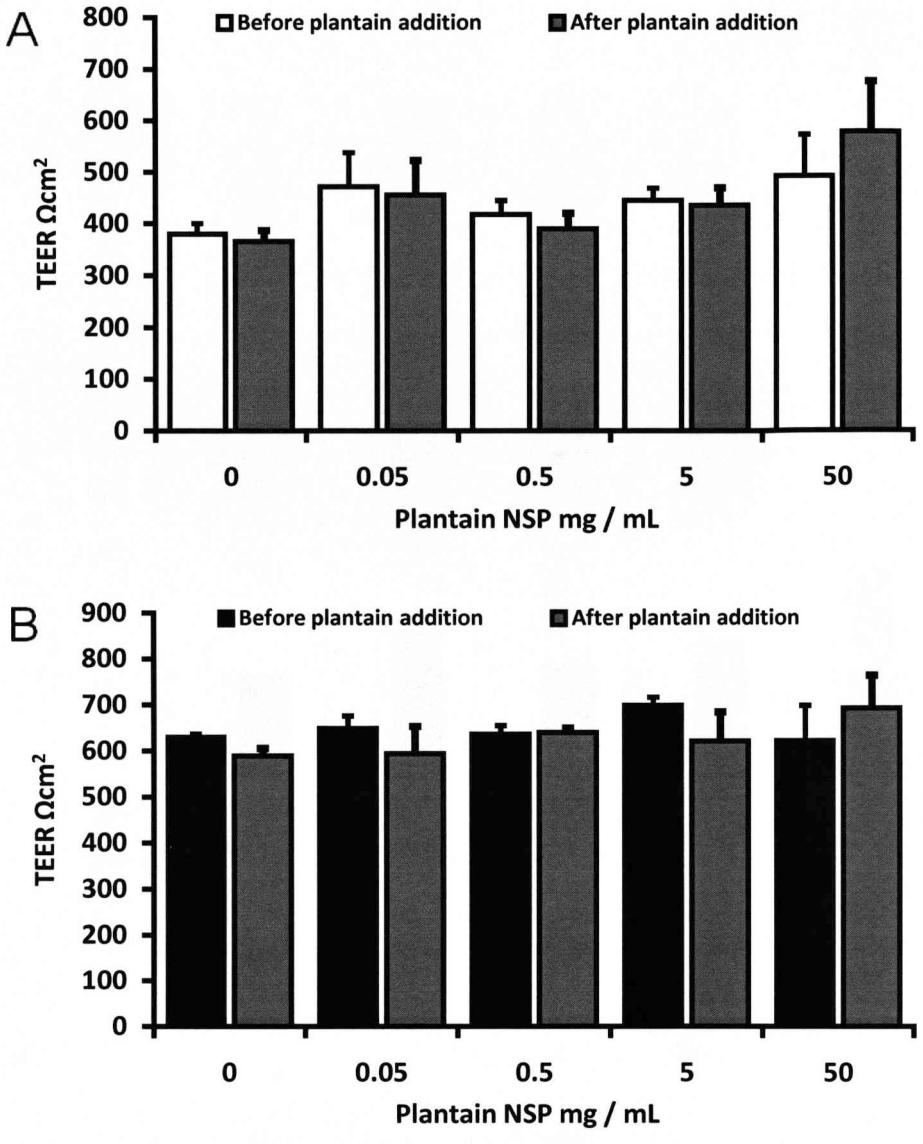


Figure 5.2 Effect of Plantain NSP upon monolayer TEER

(A) TEER for M-cell monolayers (n = 6) and (B) Caco2-cl1 monolayers (n = 3), is unaffected following 30 minute plantain incubation on the apical monolayer surface.

5.5.2 Plantain inhibits translocation of control *E.* coli K12 and Crohn's disease *E. coli* across M-cells

Translocation across M-cell monolayers was significantly inhibited by the presence of plantain at 5 mg / mL for K12 (59.0 ± 11.1 %), LF82 (61.6 ± 13.0 %), HM580 (67.4 ± 1.7 %) and HM615 (45.3 ± 3.3 %) (Figure 5.3A). As for HM605, TEER was not affected by the presence of plantain NSP in either Caco2-cl1 or M-cell monolayers. Plantain NSP, at 5 mg / mL, appears able to inhibit the translocation of some *E. coli* strains across Caco2-cl1 monolayers (Figure 5.3B). Whilst inhibition of translocation across Caco2-cl1 monolayers was observed for *E. coli* K12 (56.7 ± 13.8 %), LF82 (40.1 ± 13.0 %), HM580 (48.6 ± 14.4 %), and HM615 (80.1 ± 7.5 %), only data for HM615 demonstrated significant inhibition of translocation.

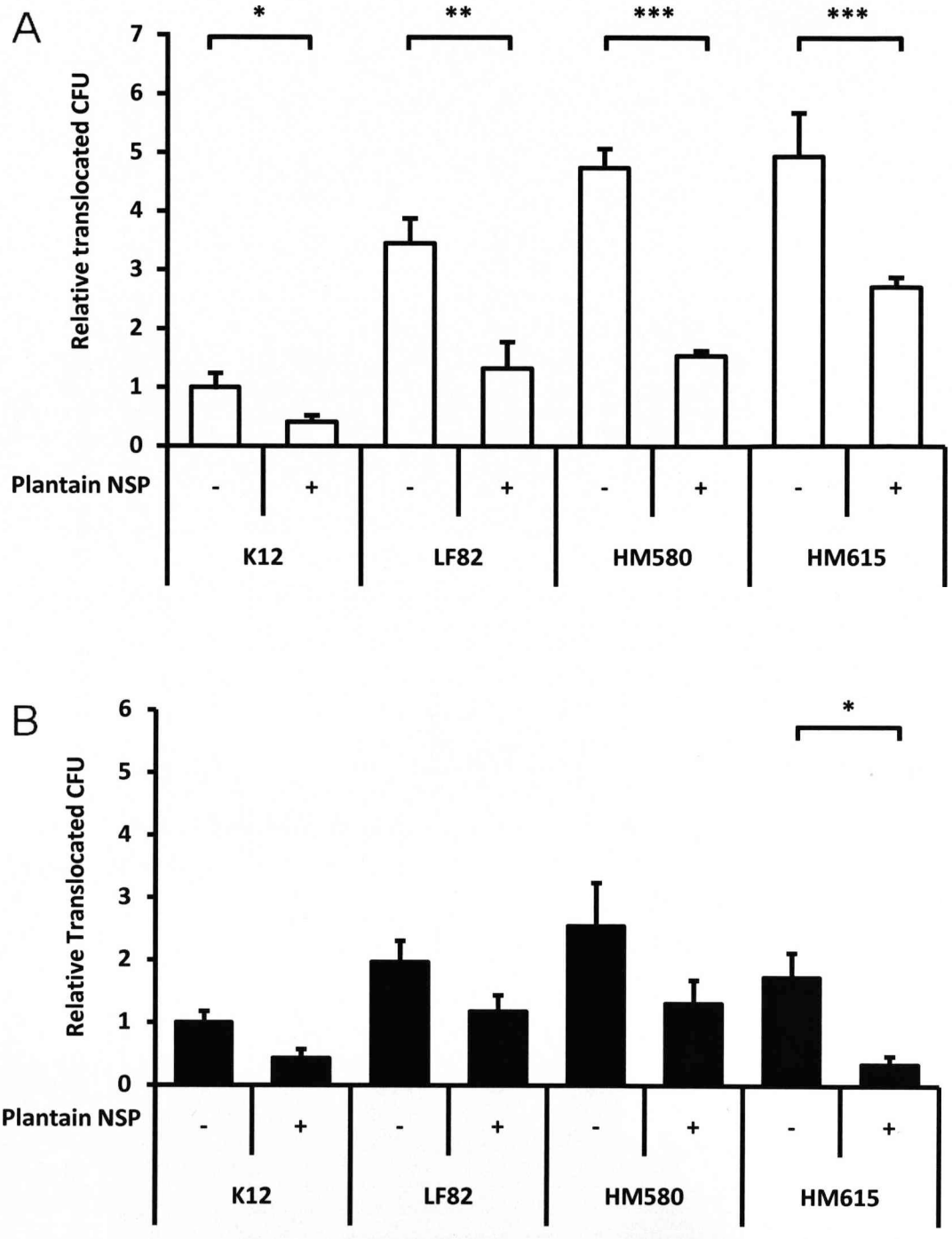


Figure 5.3 Plantain affects *E. coli* translocation across M-cell and Caco2-cl1 monolayers

(A) Plantain treatment (5 mg / mL) inhibits translocation of all *E. coli* strains across M-cell monolayers and (B) inhibits HM615 translocation across Caco2-cl1 monolayers. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ (ANOVA) (n = 3).

5.5.3 Inhibitory effect of plantain NSP is not limited to *E. coli*

The inhibitory effect of plantain upon *E. coli* translocation was found to extend to other bacterial species. Translocation of *Salmonella* Typhimurium LT2 across M-cell monolayers was inhibited by plantain at 50 mg / mL (92.8 ± 1.7 %) (Figure 5.4). Translocation of *Shigella sonnei* across Caco2-cl1 monolayers was unaffected by the presence of plantain, whilst translocation across M-cell monolayers was inhibited at a concentration of 5 mg / mL (46.4 ± 7.7 %) (Figure 5.5).

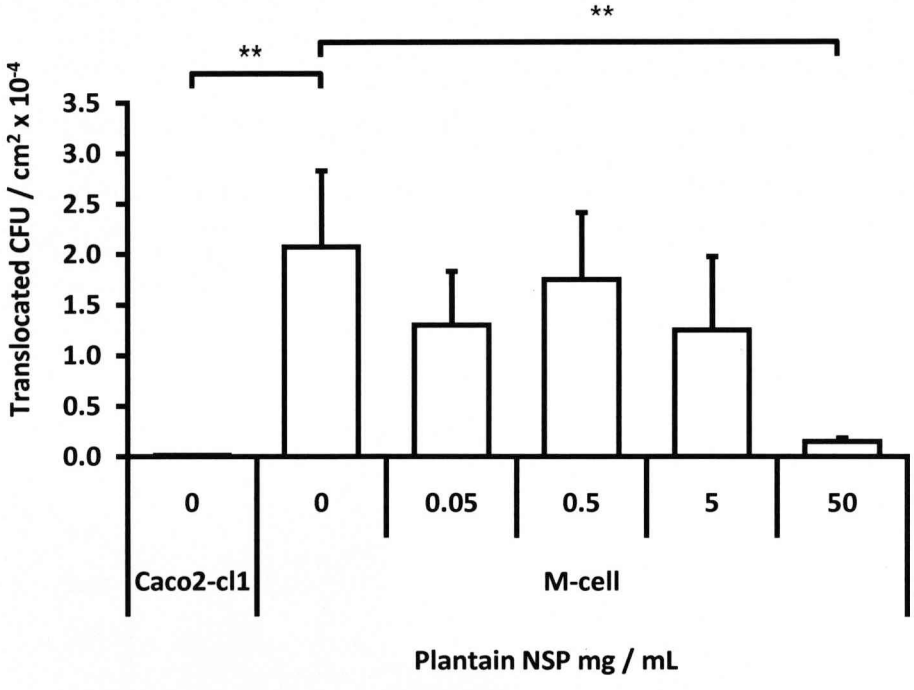


Figure 5.4 Plantain NSP inhibits *Salmonella* Typhimurium LT2 translocation across M-cells

Plantain inhibits translocation across M-cells of *Salmonella* Typhimurium LT2 at 50 mg / mL. **, $P < 0.01$ (ANOVA) (n = 5).

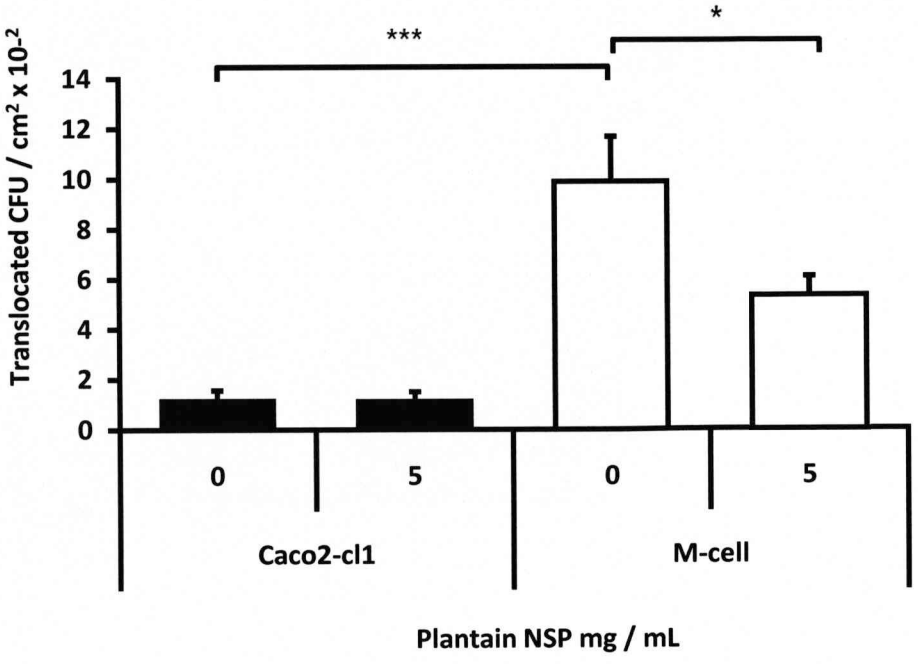


Figure 5.5 Plantain NSP inhibits *Shigella sonnei* translocation across monolayers

Plantain inhibits translocation of *Shigella sonnei* across M-cell monolayers at 5 mg / ml, but not across Caco2-cl1 cell monolayers. *, $P < 0.05$; and ***, $P < 0.001$ (ANOVA) (n = 4)

5.5.4 Broccoli NSP inhibits HM605 translocation across M-cells

Broccoli NSP was found to inhibit the translocation of HM605 across both Caco2-cl1 (Figure 5.6A) and M-cell monolayers (Figure 5.6B), in a dose dependant manner, without any significant effect upon monolayer TEER. Inhibition of translocation across Caco2-cl1 by broccoli NSP was at follows; 0.5 mg / mL, 84.5 ± 2.9 %; 5 mg / mL, 78.3 ± 11.9 %; and 50 mg / mL 75.7 ± 11.6 %. For M-cells, inhibition of translocation by broccoli NSP was 0.5 mg / mL, 49.4 ± 13.5 %; 5 mg / mL, 62.5 ± 7.3 %; and 50 mg / mL 68.7 ± 5.7 %.

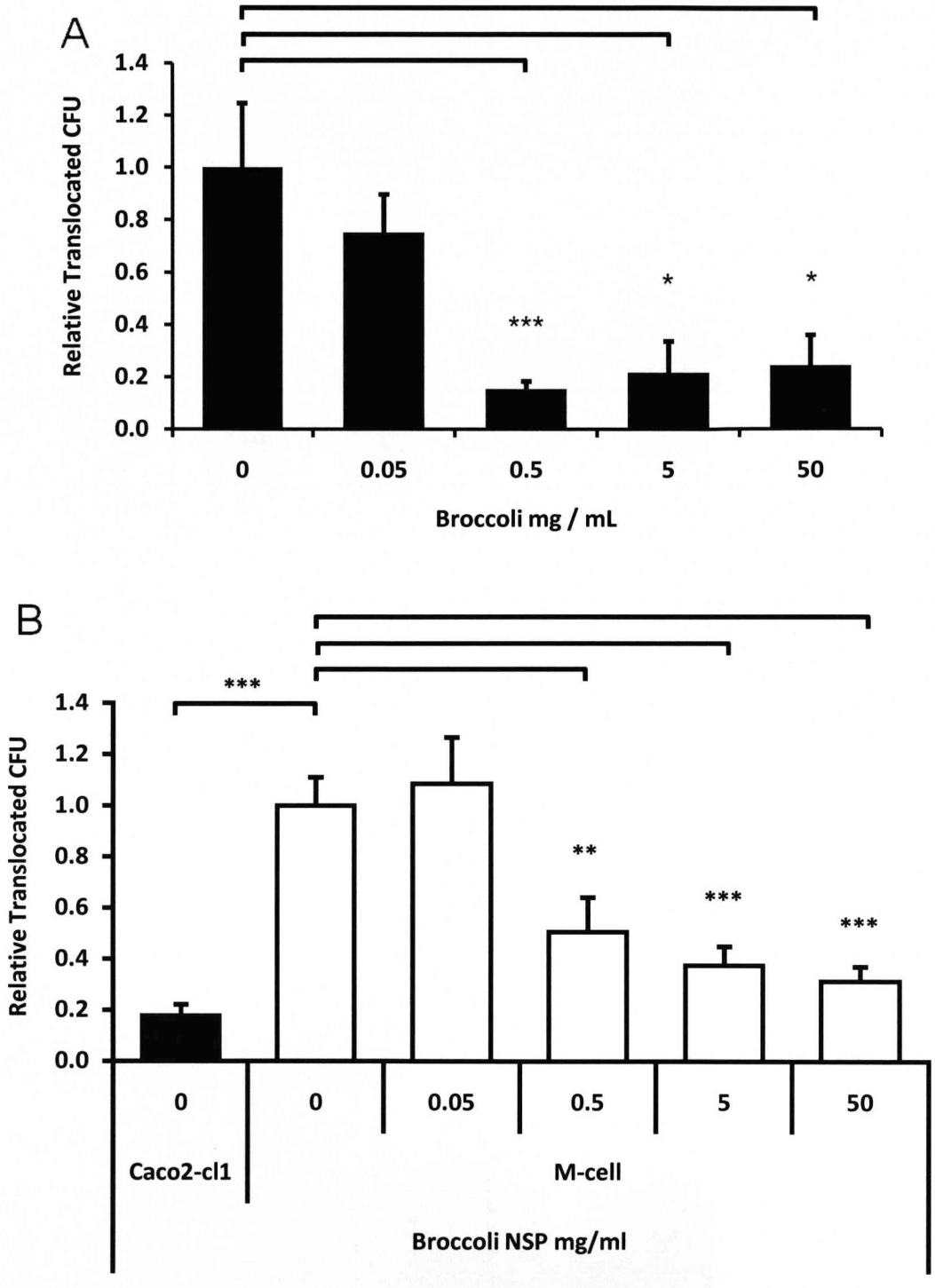


Figure 5.6 Broccoli NSP affects translocation across monolayers

HM605 translocation through Caco2-cl1 monolayers (A) and M-cell monolayers (B) is inhibited by the presence of broccoli NSP at the investigated concentrations of 0.5, 5 and 50 mg / mL. *, $P < 0.05$;**, $P < 0.01$; and ***, $P < 0.001$ (ANOVA) (n = 3).

5.5.5 Leek NSP does not inhibit HM605 translocation across M-cells

Leek NSP did not significantly affect translocation across either M-cell (Figure 5.7A) or caco2-cl1 monolayers (Figure 5.7B) at any does investigated. There was a general trend downwards indicating inhibition of translocation at higher leek NSP concentrations across M-cell monolayer, but this was not seen to be statistically significant. As with plantain and broccoli, the addition of leek NSP had no significant effect upon monolayer TEER.

5.5.6 Apple NSP does not inhibit HM605 translocation across M-cells

As with leek NSP, apple NSP was found not affect translocation of HM605 across Caco2-cl1 or M-cell monolayers (Figure 5.8), and TEER was not affected by the addition of apple NSP to the apical monolayer surface.

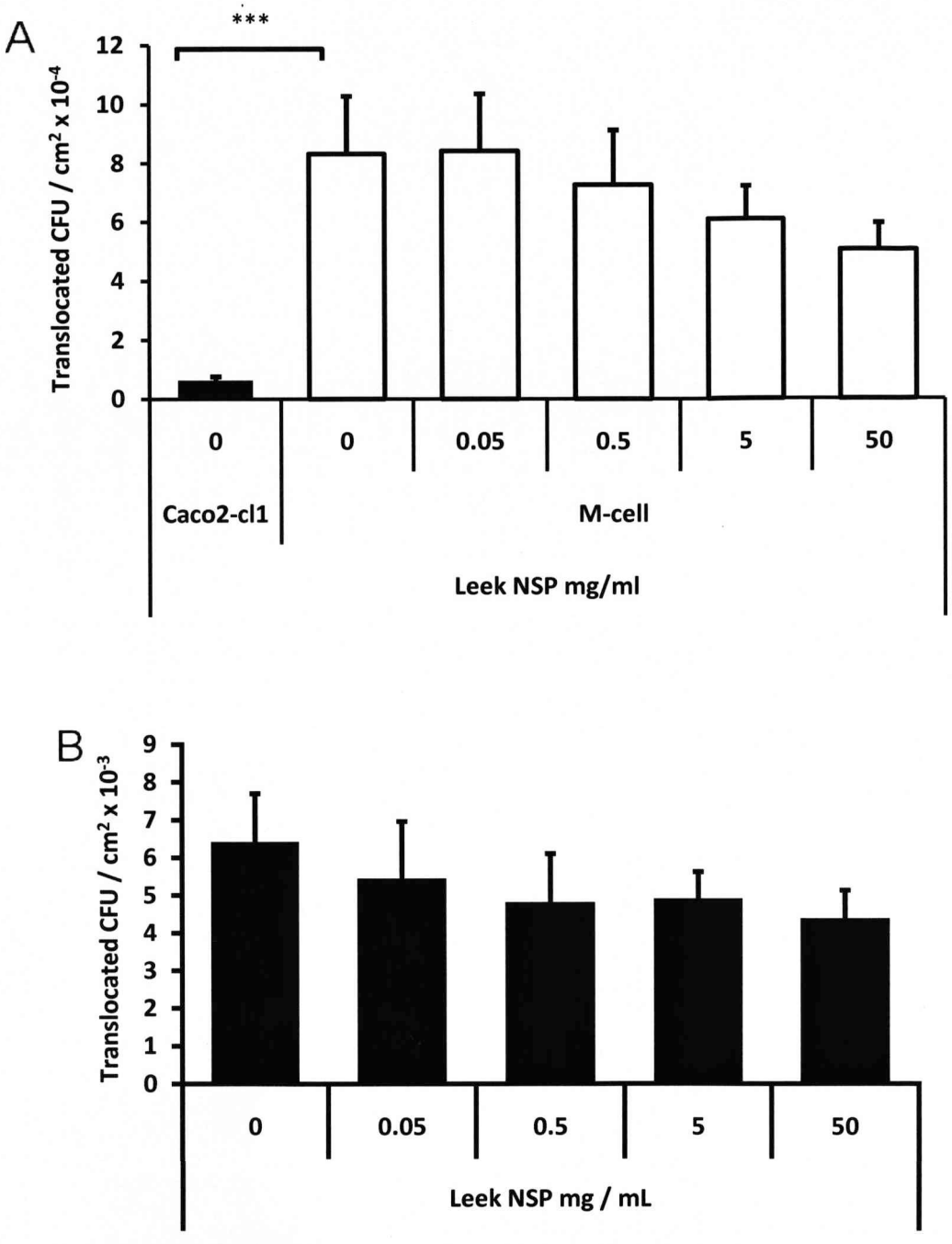


Figure 5.7 Leek NSP has little effect upon *E. coli* HM605 translocation

Leek NSP does not significantly inhibit HM605 translocation across M-cell (A) or Caco2-cl1 monolayers (B). ***, $P < 0.001$ (ANOVA) (n = 5)

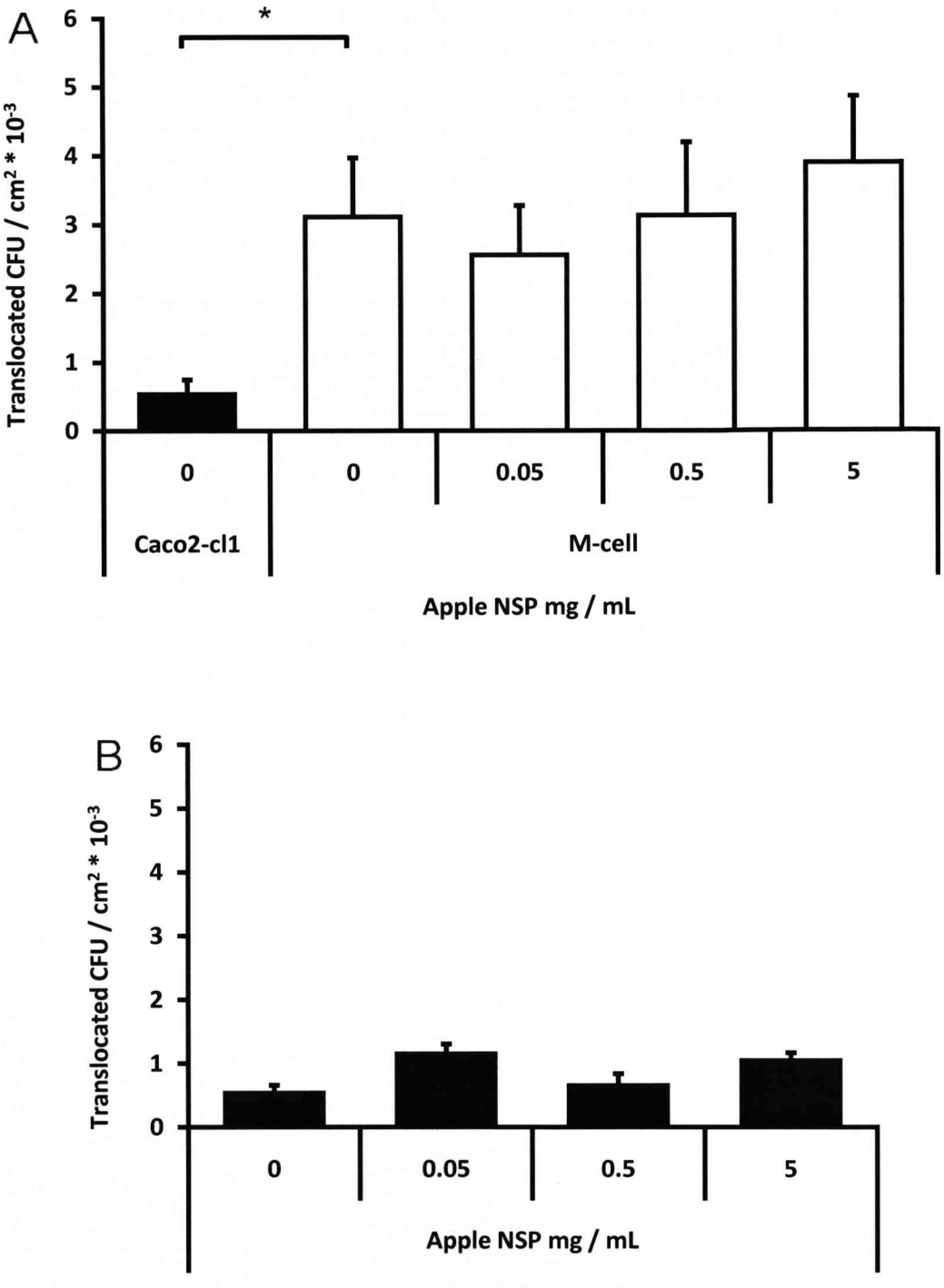


Figure 5.8 Apple NSP has little effect upon translocation

Apple NSP does not significantly inhibit HM605 translocation across M-cell (A) or Caco2-cl1 cell (B) monolayers. *, $P < 0.05$ (ANOVA) (n = 5)

5.5.7 Polysorbate-80 treatment affects translocation of HM605 across both M-cells and Caco2-cl1 cells

Polysorbate-80 treatment led to increased HM605 translocation across Caco2-cl1 and M-cell monolayers at different concentrations. Polysorbate-80 treatment at 0.01%, initially the highest concentration investigated, led to a statistically significant sixty fold increase in HM605 translocation across Caco2-cl1 monolayers (Figure 5.9A). Across M-cells, polysorbate-80 at 0.01% did not alter translocation of HM605; however, at the tenfold higher concentration of 0.1%, fivefold more HM605 were translocated across the monolayer (Figure 5.9B).

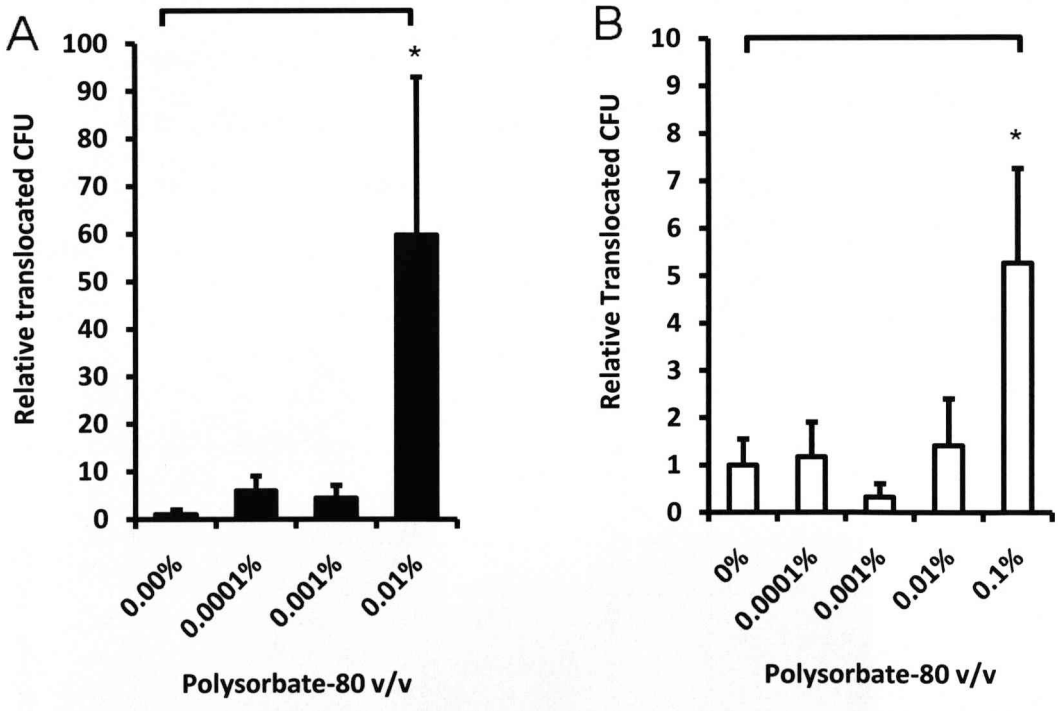


Figure 5.9 Polysorbate-80 increased *E. coli* translocation across monolayers

(A) Polysorbate-80 at 0.01% v/v causes increased translocation of *E. coli* HM605 across Caco2-cl1 monolayers. (B) Polysorbate-80 at 0.1% v/v increases translocation across M-cells cells. *, $P < 0.05$ (ANOVA) ($n = 5$).

The TEER of untreated Caco2-cl1 monolayers fell during the 30 minute incubation period in which other wells were treated with polysorbate-80. The TEER of Caco2-cl1 monolayers was significantly decreased at 0.001% and 0.01% v/v polysorbate-80, whilst TEER at 0.0001% was unaffected (Figure 5.10A). Across M-cell monolayers, there was a numerical, although not statistically significantly relevant, decrease in monolayer TEER in response to polysorbate-80 treatment at all concentrations investigated; in addition, there was a decrease in untreated M-cell monolayer TEER (Figure 5.10B).

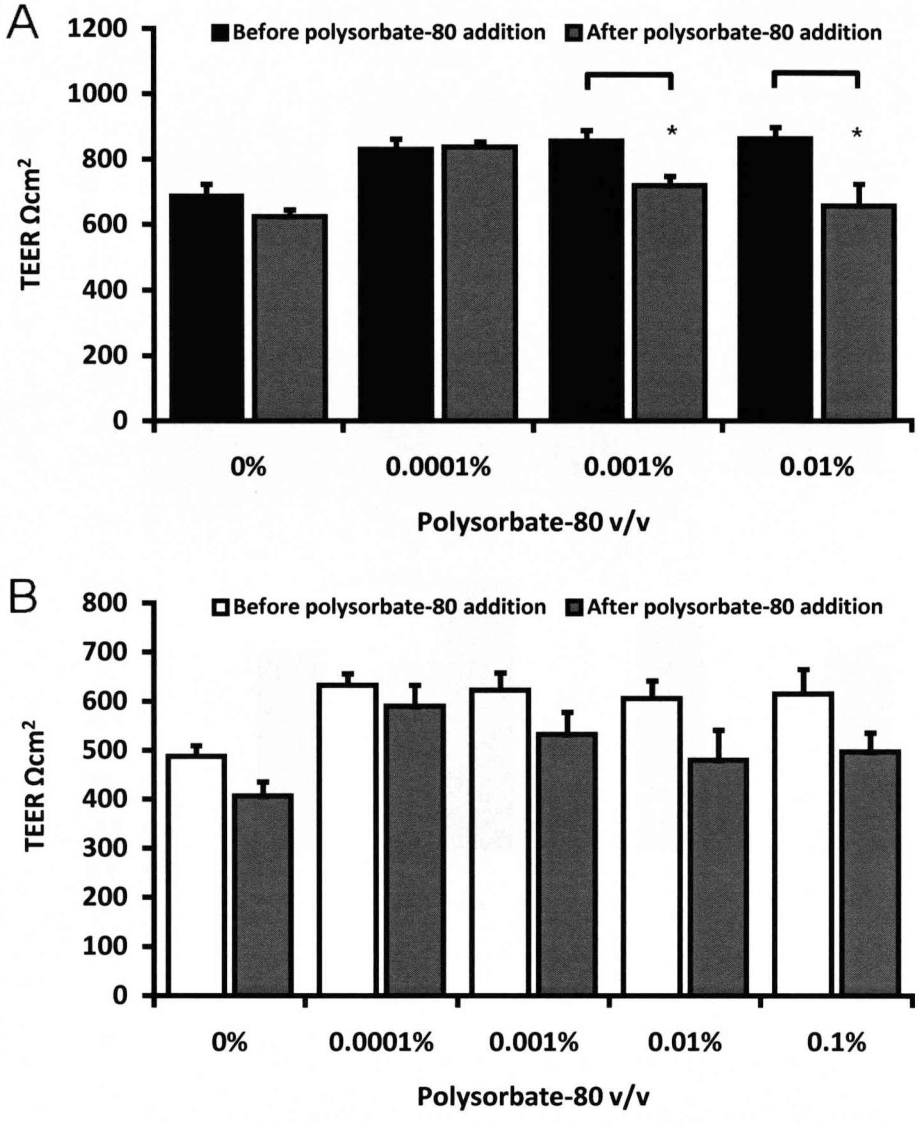


Figure 5.10 Polysorbate-80 and affect upon monolayer TEER

(A) Caco2-cl1 monolayer. Polysorbate-80 induces a decrease in Caco2-cl1 monolayer TEER at 0.01% and 0.1% v/v. However, it should be noted that there is also a decrease in TEER in the wells not receiving polysorbate-80 treatment, and whilst not statistically significant, it does cast some doubt on validity on the TEER reduction seen in the treated wells. (B) M-cell monolayer. TEER numerically decreases during polysorbate-80 incubation, although not statistically so; there is also an observable numerical decrease in TEER of the untreated M-cell monolayers. *, $P < 0.05$ (ANOVA). ($n = 5$)

5.5.8 Polysorbate-60 affects translocation of HM605 across both M-cells and Caco2-cl1 cells

For polysorbate-60, a fourteen fold increase in HM605 translocation across Caco2-cl1 monolayers was observed at 0.1% v/v (Figure 5.11A), however, no observable increase in translocation across M-cells was detected at all concentrations investigated (Figure 5.11B).

The TEER of Caco2-cl1 monolayers significantly increased in both the polysorbate-60 treated and untreated wells (Figure 5.12A); a similar, although not statistically significant pattern was observed for M-cell monolayer TEER (Figure 5.12B)

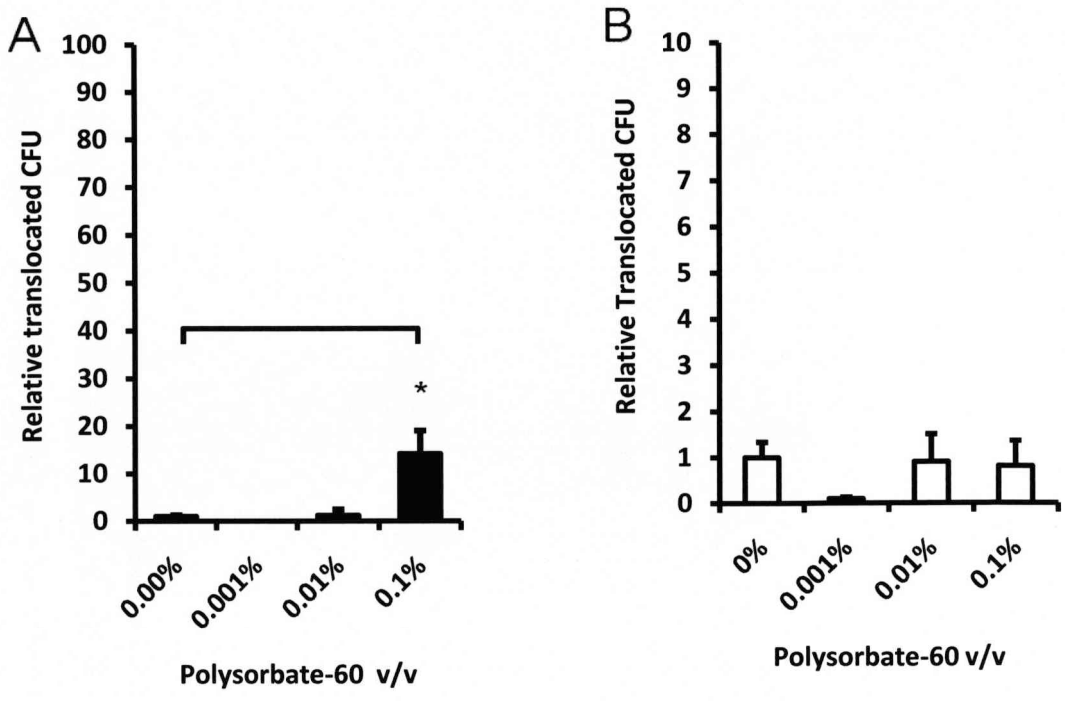


Figure 5.11 Polysorbate-60 increases *E. coli* translocation across Caco2-cl1 monolayers

Polysorbate-60 at 0. 1% v/v causes increased translocation of *E. coli* HM605 across Caco2-cl cells (A). Polysorbate-60 does not lead to increased bacterial translocation across M-cells (B). *, $P < 0.05$ (ANOVA) (n = 5).

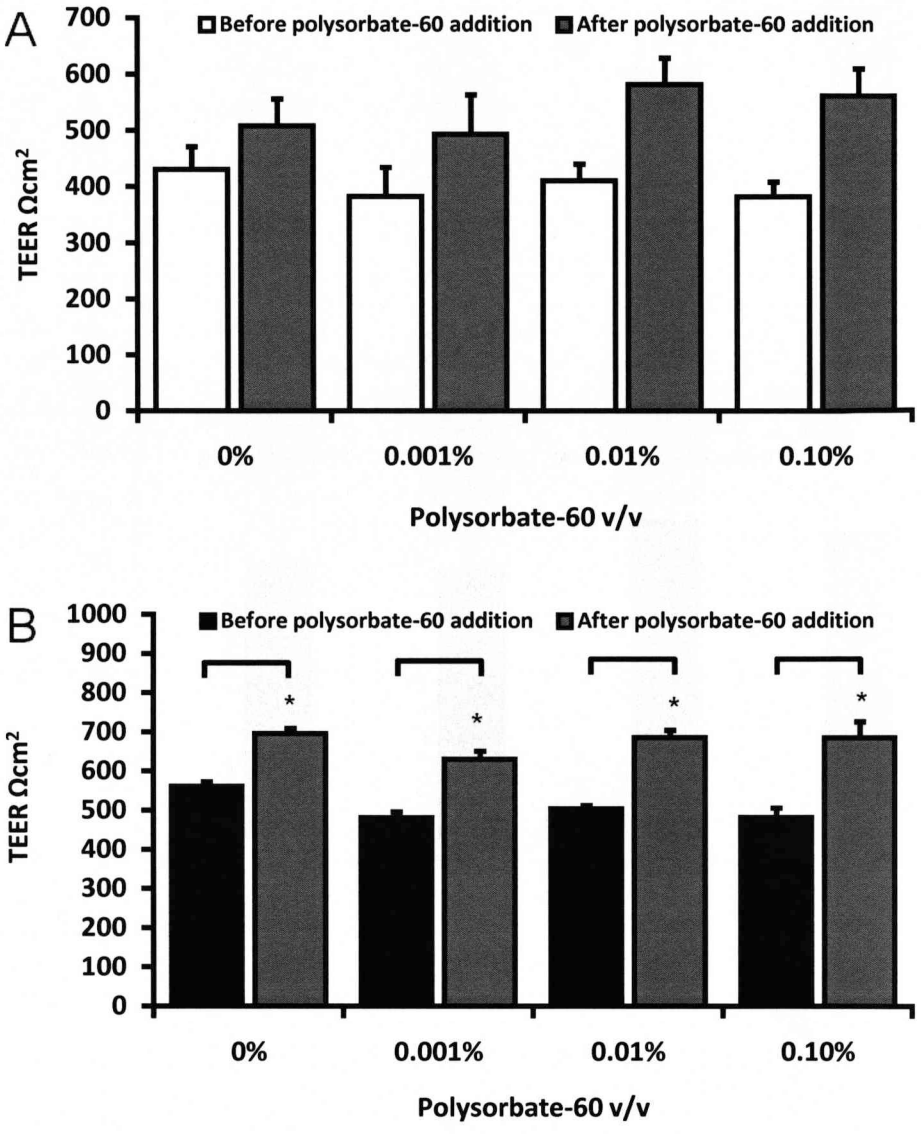


Figure 5.12 Polysorbate-60 and affect upon monolayer TEER

(A) M-cell monolayer TEER numerically increases during polysorbate-80 incubation, although not statistically so; there is also an observable numerical increase in TEER of the untreated M-cell monolayers. (B) Polysorbate-60 induces a statistically significant increase in Caco2-cl1 monolayer TEER at 0.001%, 0.01% and 0.1% v/v. However, it should be noted that there is also a statistically significant increase in TEER in the wells not receiving polysorbate-60 treatment. *, $P < 0.05$ (ANOVA) ($n = 6$)

5.6 SUMMARY OF RESULTS

1. Translocation of control *E. coli* K12, Crohn's disease AIECs LF82, HM580, HM605, HM615 and *Shigella sonnei* at 5 mg / mL, and *Salmonella* Typhimurium LT2 at 50 mg / mL, was significantly inhibited across M-cell monolayers by plantain NSP.

2. Translocation of all investigated *E. coli, Shigella sonnei* and *Salmonella* Typhimurium LT2 through control Caco2-cl1 monolayers was not affected by the presence of plantain NSP, with the notable exception of *E. coli* HM615 which was found to inhibited at the investigated concentration of 5 mg / mL.

3. Translocation of HM605 through both Caco2-cl1 and M-cell monolayers was significantly inhibited by broccoli NSP at 0.5, 5 and 50 mg / mL.

4. Translocation of HM605 across Caco2-cl1 and M-cell monolayers was not affected by the presence of either leek or apple NSP at the investigated concentrations.

5. The presence of plantain, broccoli, leek or apple NSP did not significantly affect the TEER of either Caco2-cl1 or M-cell monolayers.

6. Polysorbate-80 at 0.01% v/v and polysorbate-60 at 0.1% v/v led to increased translocation of HM605 across Caco2-cl1 monolayers; polysorbate-80 at the tenfold higher concentration 0.1% v/v led to increased translocation across M-cell monolayers, whilst polysorbate-60 treatment had no effect upon translocation at the investigated concentrations.

7. The TEER of Caco2-cl1 and M-cell monolayers did not conclusively alter as a direct result of either polysorbate-80 or polysorbate-60 treatment.

5.7 DISCUSSION

If translocation of *E. coli* across M-cells is important in Crohn's disease pathogenesis, then inhibiting this process could be of therapeutic benefit.

Inhibition of Crohn's disease *E. coli* translocation across M-cells occurred in the presence of soluble plantain NSP and soluble broccoli NSP at 5 and 50 mg / mL. In terms of an effective dose for humans, the lower concentration of 5 mg / mL soluble NSP could be readily achieved in the lumen of the human digestive tract through a daily dose of 10 g soluble NSP per day, assuming that 75% of the soluble NSP ingested is digested.

Plantain NSP at 5 and 50 mg / mL was also found to inhibit *E. coli* adhesion and invasion of Caco2-cl1 cells *in vitro*, is plausible that a diet rich in plant NSPs could inhibit bacterial adhesion to the *in vivo* epithelia, or translocation across it, as has been seen here *in vitro.* This has potential relevance not only to Crohn's disease, but other disease known or thought to be involved with irregular bacterial – host epithelial interactions, including colon cancer [Martin *et al.* 2004], or diarrhoeal disorders.

The effect of food emulsifiers on *E. coli* translocation is less clear. Polysorbate-80 at 0.1 % (v/v) led to 5-fold increased translocation across M-cells, but at the lower concentration of 0.01 % across Caco2-cl1 cells, a 54-fold increase in translocation was observed, both without any negative effect on monolayer TEER.

Increased translocation of *E. coli* in the presence of polysorbate-60 across Caco2-cl1 cells was only observed at 0.1 % (v/v), 10-fold higher than the effective concentration of polysorbate-80 causing increased translocation across the same cells.

Arguably, the relative lack of effect of polysorbates upon M-cell bacterial translocation possibly argue against a role of dietary emulsifiers in Crohn's disease. However, there increased in bacterial translocation across Caco2-cl1 cells is interesting. Polysorbate-80 has been shown to integrate within cell membranes [Zordan-Nudo *et al.* 1993] resulting in a change of membrane microviscosity [Friche *et al.* 1990]. It is possible that alterations in the membrane fluidity could alter bacterial adhesion and translocation through epithelial cells.

The data presented here indicate that Caco2-cl1 cells are more susceptible to the effects of polysorbate-80 than M-cells, and that both cell types are more susceptible to the effects of polysorbate-80 than polysorbate-60. The increase in bacterial translocation across Caco2-cl1 seen with these emulsifiers may have important implications for Crohn's disease treatment. Enteral nutritional feeding with elemental or whole protein feed as either primary or adjuvant therapy for Crohn's disease is common [Ricour *et al.* 1977; Morin *et al.* 1982], if these feeds were to contain emulsifiers they could have harmful effects when used in sick patients where intestinal permeability is already impaired.

Chapter 6

Replication of Crohn's Disease *Escherichia coli* Isolates within Macrophages and their Susceptibility to Antibiotics

6.1 HYPOTHESIS

Crohn's disease colonic mucosa-associated AIEC are internalised into, and then subsequently survive and replicate within macrophages. Antibiotics can be used to kill internalised *E. coli*.

6.2 AIMS

1. To determine survival and replication of Crohn's disease AIEC within macrophages.
2. To determine the effectiveness of clinically relevant antibiotics against internalised Crohn's disease AIEC

6.3 INTRODUCTION

In Crohn's disease tissue, the presence of *E. coli* has been most clearly observed within macrophages through the use of immunohistochemistry against an *E. coli* antigen [Liu *et al.* 1995] and by laser capture microdissection followed by DNA extraction and PCR amplification of *E. coli* DNA [Ryan *et al.* 2004]. In addition to this, *E. coli* antigens have been identified in macrophages within the lamina propria, in granulomas, and in the germinal centres of mesenteric lymph nodes in patients with Crohn's disease [Cartun *et al.* 1993; Liu *et al.* 1995]. The Crohn's disease ileal *E. coli* isolate LF82 has been shown to replicate within the mature phagolysosomes of macrophages *in vitro* [Bringer *et al.* 2006] and to induce giant cell formation [Meconi *et al.* 2007].

In controlled trials, single antibiotics such as ciprofloxacin [Prantera *et al.* 1996], metronidazole [Prantera *et al.* 1996], clarithromycin [Leiper *et al.* 2008] and rifaximin [Prantera *et al.* 2006] have showed some limited benefit as a potential Crohn's disease treatment. It is also known that metronidazole [Rutgeerts *et al.* 1995] and ornidazole [Rutgeerts *et al.* 2005] are effective against postoperative recurrence of Crohn's disease. In the clinic, corticosteroids are commonly used as an initial treatment. Studies co-administering the corticosteroid budesonide with the ciprofloxacin or metronidazole showed no improvement compared to treatment with budesonide alone [Steinhart *et al.* 2002]. Without a known cause, or primary location of activity, the choice of antibiotic treatment is currently uninformed. If Crohn's disease AIEC are found in macrophages within Crohn's disease tissue as the literature would suggest, a more targeted antibiotic treatment can be sought.

6.4 METHODS

Detailed methods for detection of AIEC replication within macrophages, and the efficacy of antibiotics against these internalised AIEC can be found within Chapter 2, as can details of TEM analysis.

Briefly, murine macrophages (J774-A1) in culture were infected with control or Crohn's disease AIEC isolates, for 2 hours, to allow bacterial internalisation, followed by extracellular gentamicin treatment for 1 hour. Macrophages were incubated for a further 3 hours to allow internalised bacteria to replicate. Cells were lysed, and fold replication calculated by overnight growth of the lysate on LB agar.

The effect of a range of antibiotics upon killing of internalised Crohn's disease AIEC HM605 was tested, both as individual antibiotics, and in combination, to establish if antibiotics could be used to eradicate Crohn's disease AIEC from infected macrophages.

6.5 RESULTS

6.5.1 Crohn's disease AIEC replicate inside J774-A1 macrophages

Following 3 hours of replication time, all seven Crohn's disease AIEC isolates tested were better able to replicate inside J774-A1 macrophages than the laboratory strain *E. coli* K12 ($P < 0.0001$, ANOVA) (Figure 6.1). The French ileal Crohn's disease AIEC LF82 replicated at a level (6.8 ± 0.8-fold) similar to that of the other seven Liverpool Crohn's disease isolates (6.36 ± 0.7-fold) in comparison to *E. coli* K12, which did not appear to replicate within macrophages (1.0 ± 0.1-fold). Six mucosal *E. coli* strains isolated from control patients (IBS / polyposis) showed greater replication (5.2 ± 0.25-fold) within macrophages than *E. coli* K12 ($P < 0.0001$, ANOVA) (Figure 6.1). On average, across all strains, these six control isolates showed significantly less replication than the eight Crohn's disease isolates tested ($P = 0.006$, Kruskal-Wallis ANOVA).

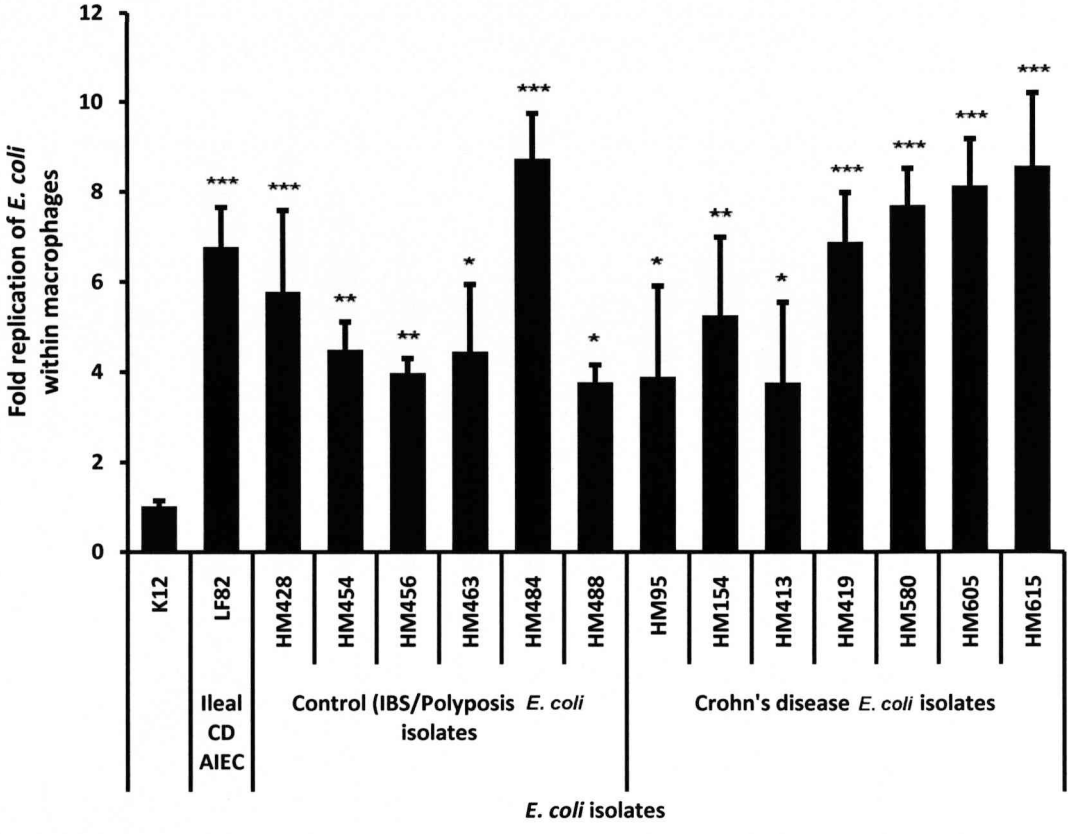


Figure 6.1 Crohn's disease and control adherent-invasive *E. coli* (AIEC) replicate effectively inside J774-A1 macrophages.

Replication of *E. coli* strains isolated from colonic Crohn's disease (n = 7) and control patients (n = 6) within J774-A1 murine macrophages (mean ± standard error of the mean) compared to the nonpathogenic reference strain *E. coli* K12. Replication is shown as the relative change after 3 h of growth within macrophages. Significant differences from *E. coli* K12 replication rates are as follows: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ (ANOVA) (n=3).

6.5.2 Crohn's disease AIEC isolates replicate within macrophage vacuoles

TEM analysis revealed the cellular morphology and intracellular structure of J774-A1 macrophages prior to infection (Figure 6.2A). Three hours after infection, HM605 was observed inside vacuoles within J774-A1 macrophages (Figure 6.2B). No internalized bacteria were observed free in the cytoplasm. The presence of bacteria within vacuoles did not affect the nuclear or cytoplasmic membrane morphology, and the macrophages retained an otherwise normal appearance. Infected cells often contained large vacuoles with the appearance of phagolysosomes (Figure 6.2C), some of which had fused, or were in the process of fusing (Figure 6.2D). Bacteria were detected that appeared to be undergoing replication (Figure 6.2E and Figure 6.2F). Upon closer inspection, it was observed that HM605 reside in membrane bound vacuoles within J774-A1 cells (Figure 6.3).

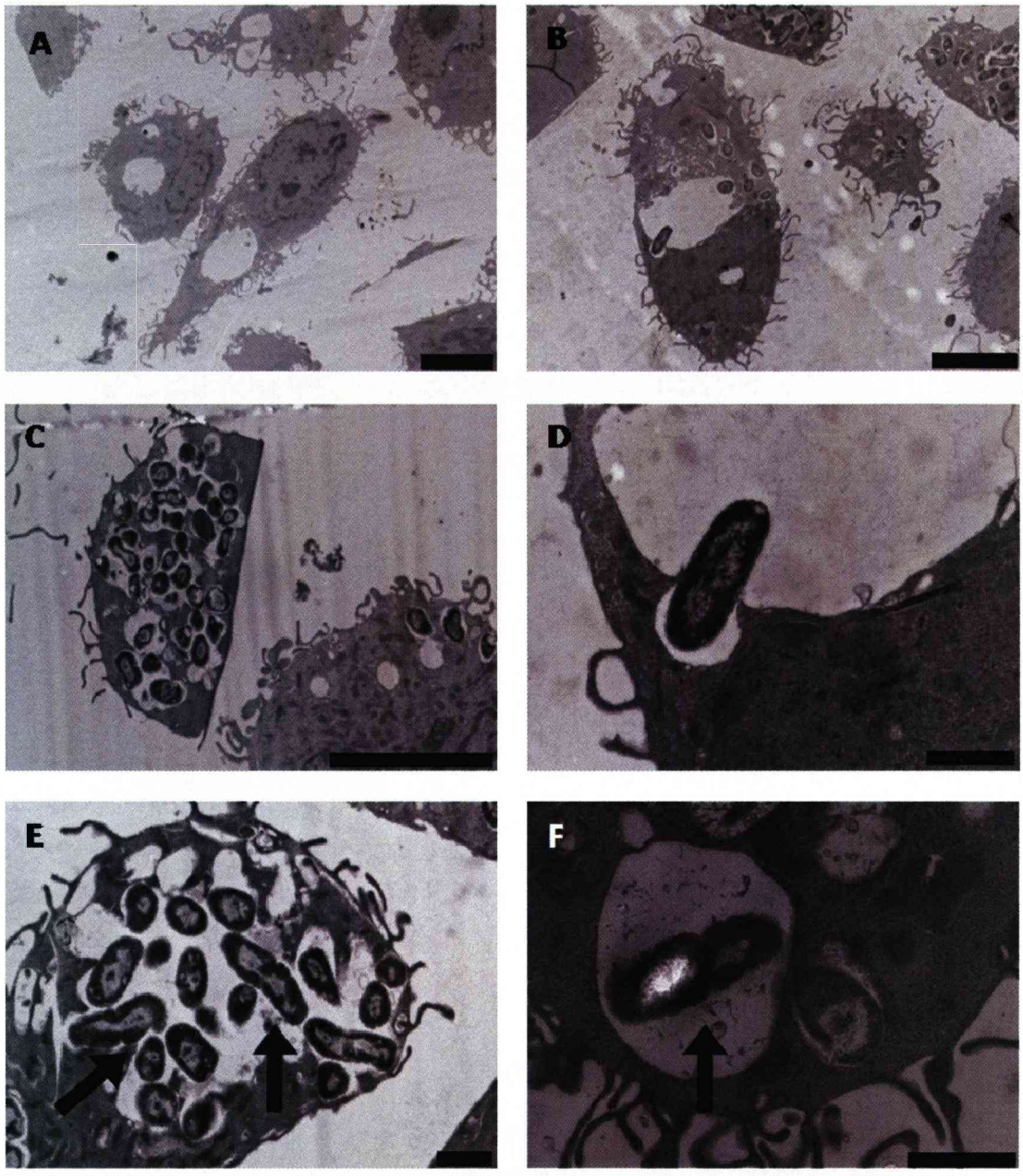


Figure 6.2 Crohn's disease AIEC HM605 replicates within vacuoles of J774-A1 cells.

(A) TEM analysis reveals that uninfected J774A.1 cells have large vacuoles, with a regular cell surface membrane, containing frequent membrane protrusions. (B) Cells infected with Crohn's disease AIEC HM605 retain this regular structure; internally, the HM605 can be found within both large and small vacuoles. (C) Heavily infected cells contained multiple vacuoles which resemble phagolysosomes. (D) Small vacuoles containing HM605 fused with much larger vacuoles to form phagolysosomes. (E) Crohn's disease AIEC HM605 replicates within structures which resemble phagolysosomes (filled arrows). (F). Crohn's disease AIEC HM605 also replicates within smaller vacuoles (filled arrows). A – C, Bar = 5μm; D – F, Bar = 1μm.

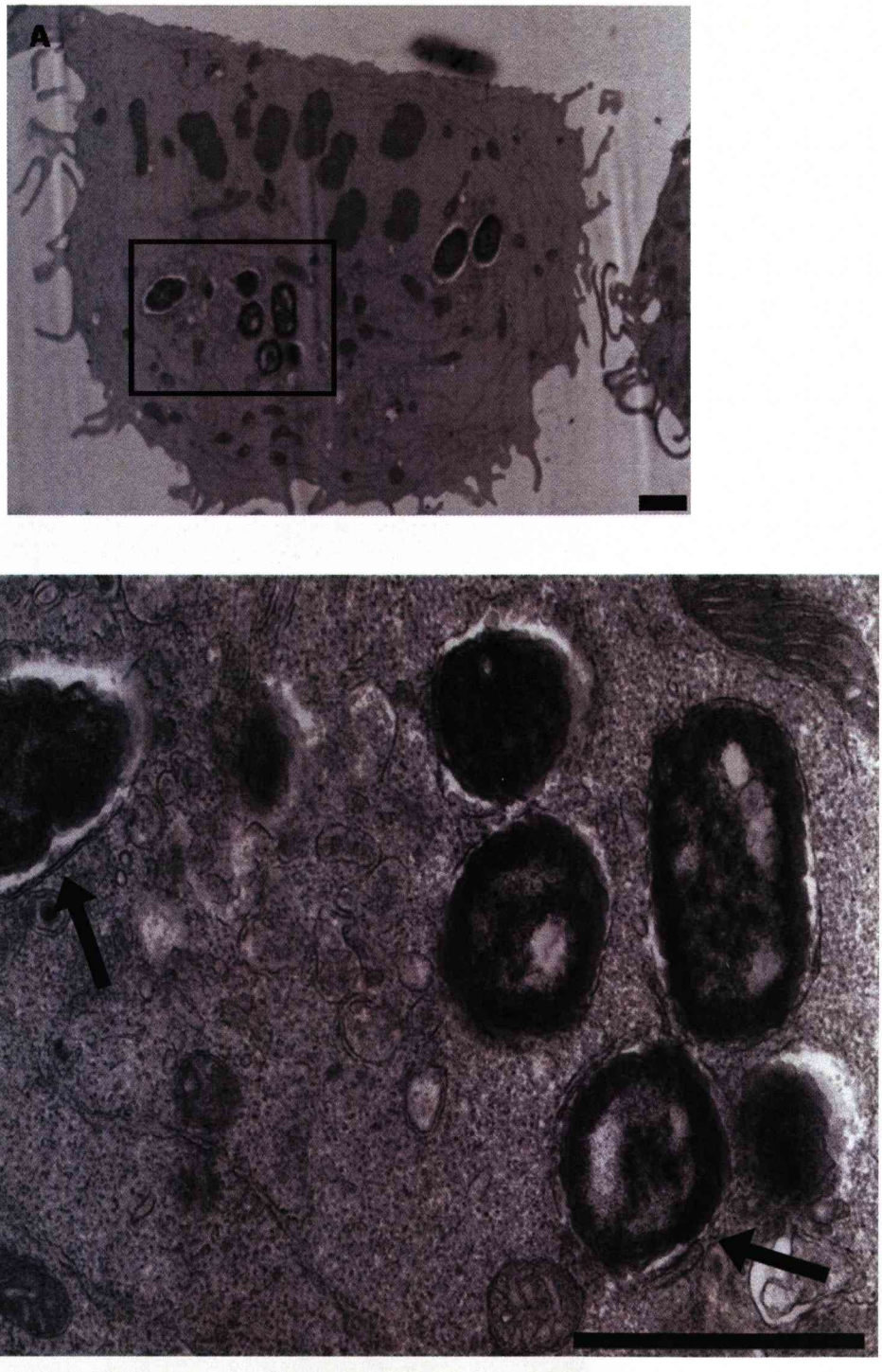


Figure 6.3 Crohn's disease AIEC HM605 is found in membrane bound vacuoles.

(A) Low magnification image of HM605 within J774-A1 macrophages. The black box represents the higher magnification image seen in figure B. (B) High magnification image of HM605 within membrane bound vacuoles (black arrows). Bar = 1μm.

6.5.3 Susceptibility of Crohn's disease AIEC HM605 within J774-A1 cells to single antibiotics

The effect of a range of antibiotics upon killing of internalised HM605 in J774-A1 was investigated at C_{max}, the commonly observed peak serum concentration seen in patients receiving conventional doses of each antibiotics, and at 10% of C_{max}. The commonly observed C_{max} values seen in patients receiving conventional doses of the antibiotics used in this study are listed in Table 6.1 (modified from [Subramanian *et al.* 2008]).

At C_{max}, significant intracellular killing was observed for all antibiotics investigated, with the exception of gentamicin, which did not lead to significant killing of internalised HM605, but which did kill all extracellular HM605 (100 ± 0 % killing in 3h). The most potent antibiotic was found to be ciprofloxacin (99.5 ± 0.3 % killing); TEM analysis revealed internalised HM605 in the process of being killed (Figure 6.4).

Table 6.1 Typically observed C_{max} values seen in patients receiving conventional doses of antibiotics, and the efficacy of these antibiotics against internalised Crohn's disease AIEC HM605 in J774-A1 macrophages.

Antibiotic	Dose (mg [route])[a]	Cmax (μg/ml)[b]	Reference	% Intracellular HM605 killing at C_{max} (mean ± SEM)[c]
Ciprofloxacin	400 (i.v)	4	[Saravolatz *et al.* 2005]	99.5 ± 0.3
Clarithromycin	500 (p.o)	3	[Lohitnavy *et al.* 2003]	73.4 ± 3.2
Rifampin	600 (p.o)	10.5	[Peloquin *et al.* 1999]	94.4 ± 1.0
Tetracycline	250 (p.o)	1.7	[Ochs *et al.* 1978]	55.9 ± 4.4
Trimethoprim	160 (p.o)	2.3	[Sturgill *et al.* 1999]	64.3 ± 8.4
Gentamicin[d]		18	[Gillbert 2000]	7.8 ± 15.2

[a] p.o., orally; i.v., intravenously.

[b] Concentrations corresponding to the peak serum levels of the respective antibiotics following administration of conventional doses in humans based on published literature – see [Subramanian *et al.* 2008].

[c] All antibiotics show significantly more HM605 killing compared to antibiotic-free survival rates, $P < 0.001$ (ANOVA). This single antibiotic data at C_{max} is the work of Dr Sreedhar Subramanian, University of Liverpool, Liverpool, UK [Subramanian *et al.* 2008].

[d] Included in table for comparison purposes. Gentamicin was used to kill extracellular at 20 μg / mL without affecting internalised HM605.

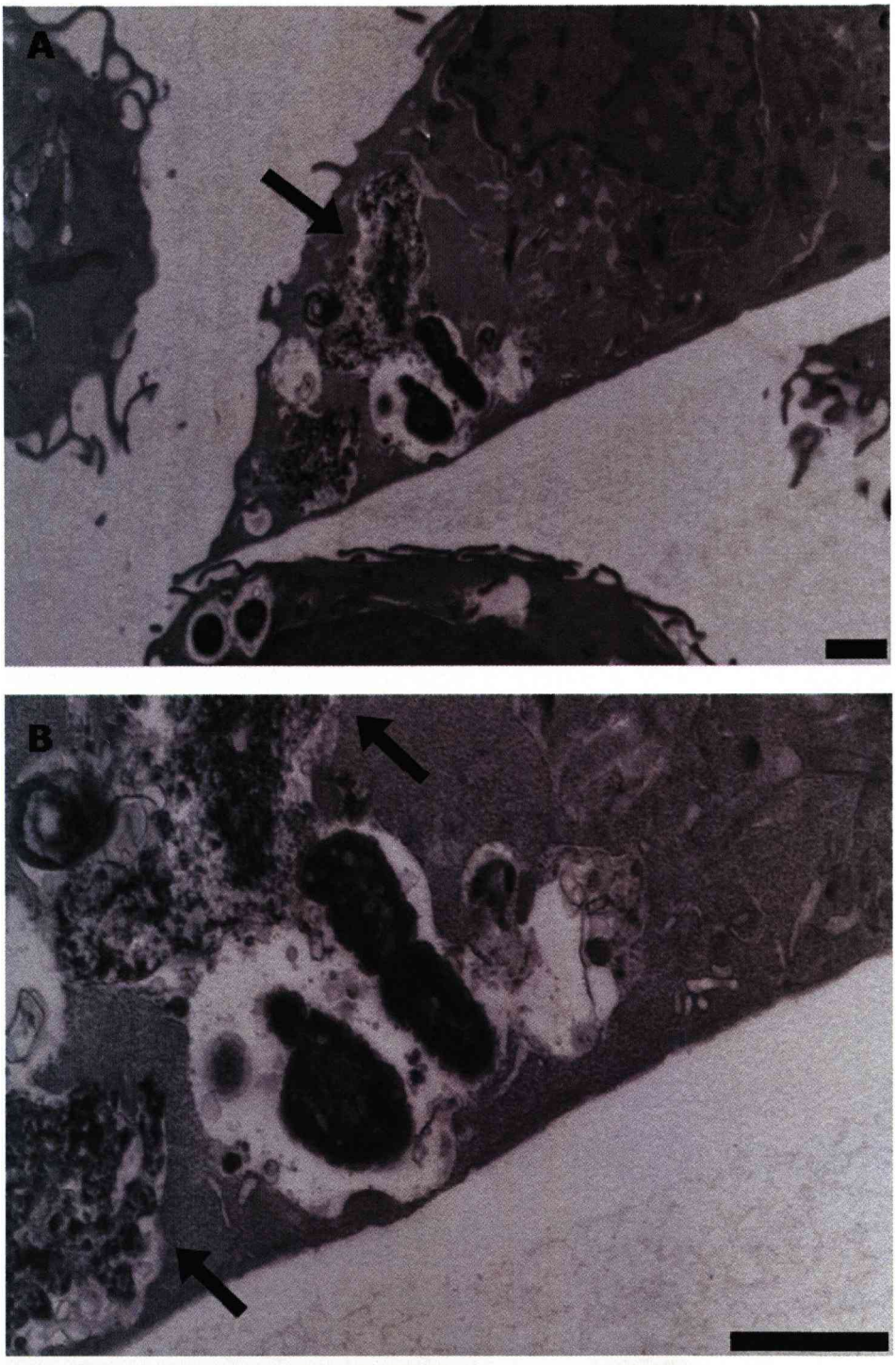


Figure 6.4 Internalised Crohn's disease AIEC HM605 within J774-A1 macrophages, treated with ciprofloxacin, apparently in the process of being killed.

(A) low power magnification and (B) high power magnification of HM605 undergoing killing within J774-A1 macrophages (black arrows) as a result of ciprofloxacin treatment at 0.1 µg / mL for 3 hours following an initial 3 hour infection period, as indicated by the presence of dark granular matter within the HM605 containing vacuole. Bar = 1 µm

6.5.4 Susceptibility of Crohn's disease AIEC HM605 within J774-A1 cells to combination antibiotics

Combination antibiotic treatment at C_{max} did not lead to significantly higher killing than single antibiotics treatment (ciprofloxacin alone, 99.1 ± 0.2 % killing; ciprofloxacin, trimethoprim and tetracycline, 99.1 ± 0.3 % killing; ciprofloxacin, trimethoprim and rifampin, 99.6 ± 0.1 % killing, mean ± SEM) (Figure 6.5).

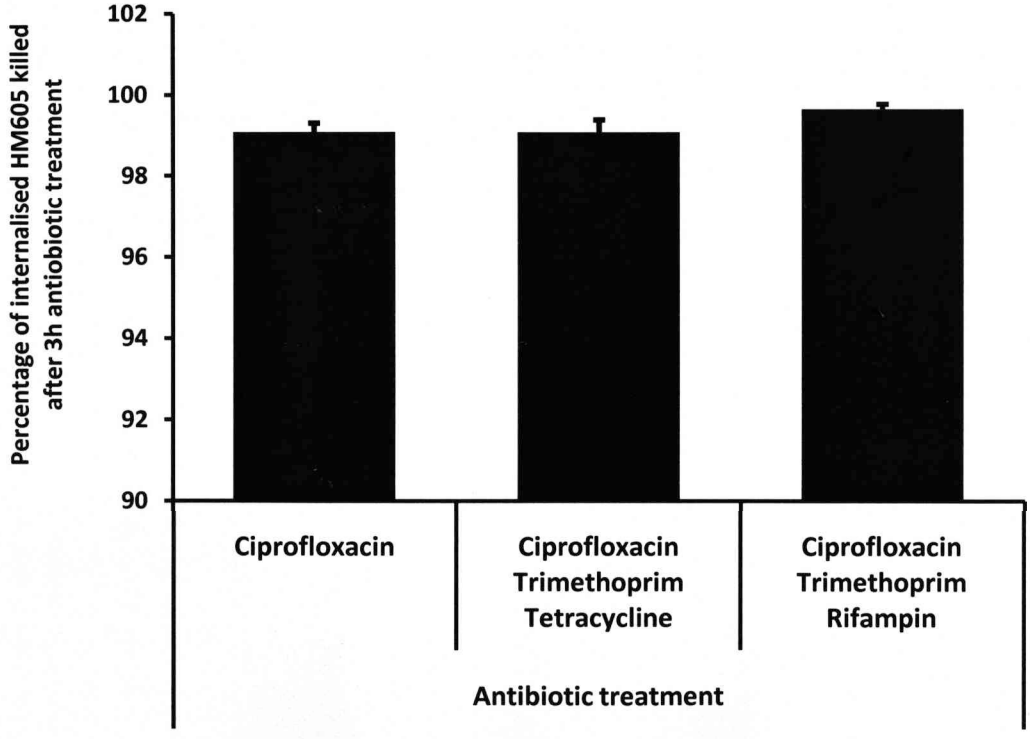


Figure 6.5 Antibiotic combinations at C_{max} do not result in significantly higher killing of internalised HM605.

When antibiotics are used at C_{max} there is no increase in killing of internalised HM605 when ciprofloxacin is used alone, or when used in combination with trimethoprim and tetracycline or rifampin. (n = 4)

Triple antibiotic treatment at the highest concentrations used did not lead to toxic effects upon the macrophages any more so than single antibiotic treatment, as estimated by viable macrophage cell counting (Figure 6.6).

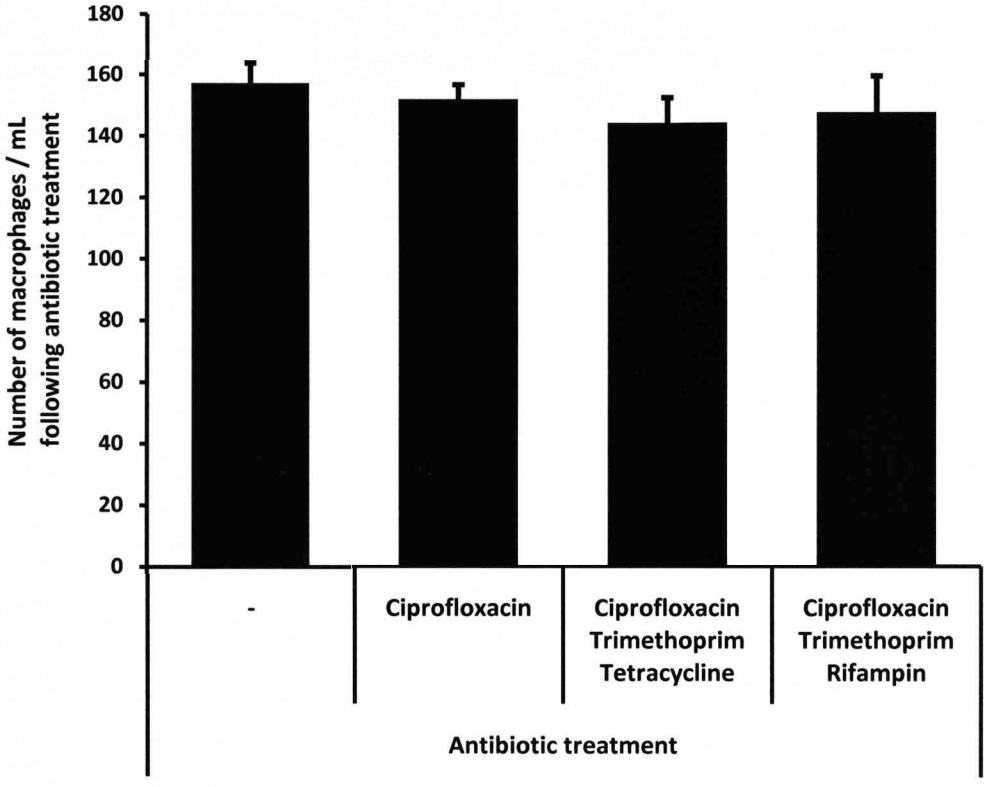


Figure 6.6 Triple antibiotic administration to macrophages does not lead to a decrease in the number of viable cells

Without antibiotic treatment, there were 157.3 ± 6.4 macrophages / mL; the addition of a single antibiotic at C_{max} resulted in 152 ± 4.7 macrophages / mL, whilst the use of the triple antibiotic combination ciprofloxacin, trimethoprim and tetracycline at C_{max} led to 144.3 ± 8.2 macrophages / mL; ciprofloxacin, trimethroprim and rifampin at C_{max} led to 147.5 ± 11.7 macrophages / mL, so no differences were observed. (n = 3)

At 10% of C_{max}, four different antibiotic combinations were investigated, using 5 different antibiotics. There was no difference in killing between the single antibiotic ciprofloxacin (92.4% ± 0.9% killing) and the combination treatment consisting of ciprofloxacin, trimethoprim and rifampin (94.0% ± 1.2% killing). Significantly higher levels of HM605 killing were observed with double antibiotic treatment, consisting of ciprofloxacin and tetracycline (96.4% ± 0.7% killing, $P < 0.05$ ANOVA). In the triple antibiotic treatment groups, significantly higher killing was seen for ciprofloxacin, tetracyclin and clarithromycin (98.3% ± 0.2%; $P < 0.01$ (ANOVA)) and for ciprofloxacin, tetracycline and trimethoprim (97.2% ± 0.4%; $P < 0.05$ (ANOVA)) (Figure 6.7).

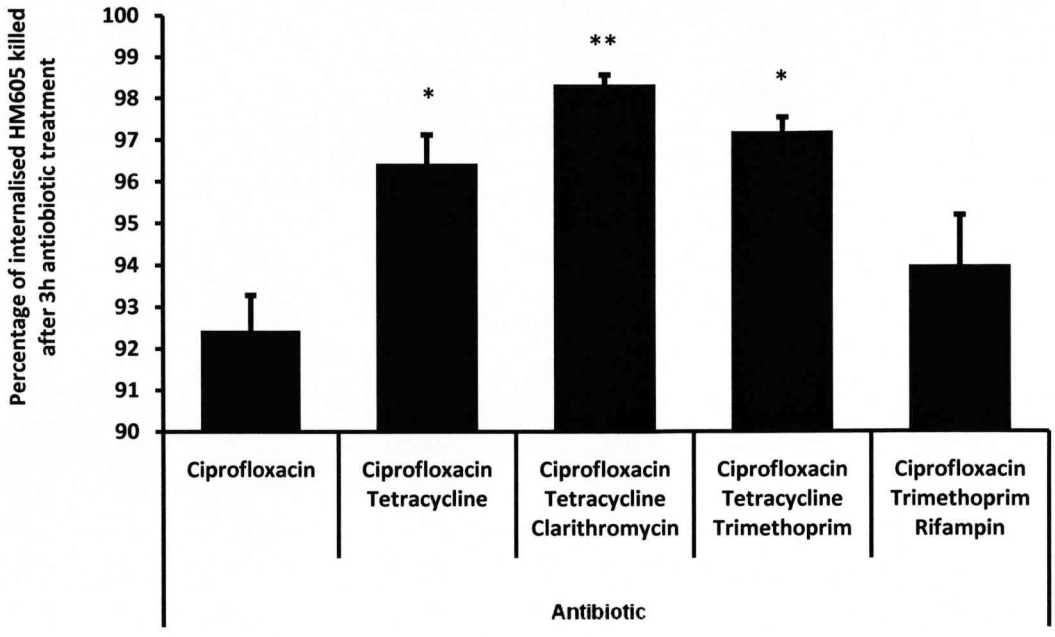


Figure 6.7 At 10% C_{max}, intracellular killing of HM605 can be increased by using combination antibiotic treatment.

At 10% C_{max}, intracellular killing of HM605 by ciprofloxacin was 94.1% ± 0.8%. In all cases, the addition of the antibiotic tetracycline led to significantly higher killing of HM605 than when ciprofloxacin was used alone; ciprofloxacin with tetracycline, 96.4% ± 0.7%; ciprofloxacin with tetracycline and clarithromycin, 98.3% ± 0.2%; ciprofloxacin with tetracycline and trimethoprim, 97.2% ± 0.4%. Treatment with ciprofloxacin, trimethoprim and rifampin (94.0% ± 1.2%) did not lead to an increased in HM605 killing compared to milling with ciprofloxacin alone. *, $P < 0.05$; and **, $P < 0.01$ (ANOVA) (n=4)

6.6 SUMMARY OF RESULTS

1. The six control patient AIEC and seven Liverpool colonic mucosa-associated Crohn's disease AIEC are better able to survive and replicate within J774-A1 macrophages than the laboratory strain *E. coli* K12, as is the ileal Crohn's disease AIEC LF82.

2. AIEC HM605 survives and replicates within membrane bound structures which resemble phagolysosomes.

3. At C_{max}, combination antibiotic treatment using ciprofloxacin, trimethoprim and either tetracycline or rifampin confers no increase in bacterial killing compared with ciprofloxacin treatment alone.

4. At 10% C_{max}, using ciprofloxacin with tetracycline leads to increased bacterial killing compared to ciprofloxacin treatment alone. The most effective intramacrophage killing of HM605 at 10% C_{max} was seen using the antibiotic combination of ciprofloxacin with tetracycline and clarithromycin.

6.7 DISCUSSION

The colonic mucosa-associated Crohn's disease AIEC described here are able to survive and replicate more readily than the laboratory control *E. coli* K12. In addition these AIECs are also able to replicate within vacuoles within macrophages, without any apparent toxic effect on the macrophage itself. In this respect, the colonic mucosa-associated AIEC described here are similar in that they replicate at a level already observed previously with the

characterised ileal Crohn's disease *E. coli* isolate LF82 [Glasser *et al.* 2001]. In addition, the colonic mucosa-associated Crohn's disease AIEC replicate within structures which resemble phagolysosomes [Subramanian *et al.* 2008], as previously reported for LF82 [Bringer *et al.* 2006].

As with Crohn's disease AIEC, AIEC from healthy control patients (isolated from IBS / polyposis colonic mucosa tissue) are able to survive and replicate better within macrophages than *E. coli* K12. This could be accredited to a comparatively unaggressive phenotype of *E. coli* K12; however there is extensive overlap between replication rates of Crohn's disease AIEC and control patient AIEC within macrophages, demonstrating that individual strains of AIEC do not absolutely correspond to their disease / healthy status in terms of replication inside macrophages *in vitro*. *In vivo,* however, *E.* coli have been identified within the macrophage tissue of Crohn's disease by immunohistochemistry [Liu *et al.* 1995], and by PCR demonstration of ribosomal DNA in laser-dissected granulomas [Ryan *et al.* 2004], but have not been identified in healthy control patient macrophage tissue.

These finding here raise the possibility that, *in vivo*, it is not the pathogenic nature of the AIEC themselves which cause an increased presence in Crohn's disease macrophages, but rather a problem with the macrophages of Crohn's disease being unable to clear the bacterial contents, which does not occur in healthy individuals.

Indeed, macrophages are not the first line of defence in fighting bacterial species which have escaped the intestinal lumen and breach the intestinal barrier [Allen 2003]. These bacteria will first come into contact with various antimicrobial peptides, such as defensins [Wehkamp *et al.* 2005; Nuding *et al.* 2007], and then with neutrophils [Allen 2003], and subsequently, bacteria

which had evaded elimination would come into contact with macrophages, with are generally poorer at bacterial killing than neutrophils [Allen 2003].

In Crohn's disease, there is some evidence that the tissue itself has lower antimicrobial activity [Nuding *et al.* 2007]. There is also evidence of neutrophil dysfunction in Crohn's disease; following trauma to the rectum, ileum or skin, there is abnormally low neutrophil accumulation and a reduction in IL-8 production [Marks *et al.* 2006]. In addition, the rare inherited conditions chronic granulomatous disease [Huang *et al.* 2004] and glycogen disease type 1b [Couper *et al.* 1991] are both associated with well characterised defects in bacterial killing by neutrophils, and in addition, are associated with Crohn's disease-like intestinal disease.

If neutrophils fail to clear AIEC, it is likely that those AIEC would be taken up by intestinal macrophages, where they have previously been seen *in vivo* [Liu *et al.* 1995; Ryan *et al.* 2004]. The presence of AIEC within macrophages could then initiate an inflammatory cascade, resulting in either elimination of the AIEC, or further facilitating bacterial invasion [Aderem 2003]. If, *in vivo*, the AIEC are not killed by the macrophages, and do not induce apotosis or macrophage toxicity, as the *in vitro* data presented here and elsewhere would suggest [Glasser *et al.* 2001], it is possible that a chronic granulomatous reaction, typical of that seen in Crohn's disease, would result.

Successful eradication of bacteria by antibiotics is dependent on a number of pathogen-, drug-, and patient-related factors. For the antibiotics tested here, efficacies against Crohn's disease *E. coli* isolates at C_{max} as well as at 10% C_{max} were assessed. Ciprofloxacin, clarithromycin, tetracycline, and trimethoprim exhibit significant bacterial killing of AIEC internalised in macrophages at much lower concentrations than C_{max}. Apart from ciprofloxacin, none of the

other antibiotics studied achieved close to complete killing at conventionally seen C_{max} levels. When tested at 10% C_{max}, ciprofloxacin exhibited significant antibacterial effects, which were compounded when the antibiotic was used in conjunction with tetracycline, or with tetracycline and clarithromycin or trimethoprim.

Based on the data presented here, clinical trials in Crohn's disease using combinations of antibiotics could be possible. The combination of antibiotics chosen should probably include ciprofloxacin because of its superior *in vitro* efficacy, with perhaps tetracycline, as these two antibiotics in combination led to significant bacterial killing at 10% C_{max}, more so than ciprofloxacin alone. Even though there are theoretical objections to using a bacteriostatic antibiotic (tetracycline) in combination with bactericidal antibiotics (ciprofloxacin), doxycycline-rifampin combinations have been used with good effect in treating brucellosis [Agalar *et al.* 1999]. However, is should be acknowledged that antibiotic combinations could lead to an increase of resistant bacterial strains, and the reduction of some possibly beneficial bacteria.

Chapter 7

Summary of Key Findings

7.1 SUMMARY OF KEY FINDINGS

1. Control patient and Crohn's disease AIEC invade into Caco2 and Caco2-cl1 cells to different extents. Invasion does not correlate with disease status of the patient from whom the isolates were derived. Some AIEC strains invade Caco2 cells to a lesser extent than control *E. coli* K12; all AIEC invade Caco2-cl1 cells to a greater extent than *E. coli* K12.

2. Plantain NSP at 0.5, 5 and 50 mg / mL inhibits *E. coli* invasion into Caco2-cl1 cells, and at 5 and 50 mg / mL, inhibits *E. coli* adhesion to Caco2-cl1 cells.

3. *In vitro* derived M-cells have a varied expression of apical microvilli, unlike Caco2-cl1 cells which have a regular expression of apical microvilli. M-cells have increased binding of *Aleuria aurantia* lectin, and decreased expression of alkaline phosphatase compared with Caco2-cl1 monolayers.

4. M-cell monolayers translocate *Salmonella* Typhimurium LT2 and *Shigella sonnei* 80-fold and 8-fold more than Caco2-cl1 monolayers respectively, independently of monolayer TEER.

5. HM605 translocation across M-cell monolayers (when TEER > 300 Ωcm^2) is just detectable at 15 minutes, and then increases steadily over time, without significant disruption to the monolayer integrity. Translocation across Caco2-cl1 cells was just detectable after 30 minutes, and again, translocation increased over time. Translocation was not dependent upon TEER when TEER > 300 Ωcm^2.

6. HM605 was detected within cells of M-cell monolayers by TEM, but not in Caco2-cl1 monolayers, or between cells.

7. Translocation of *E. coli* K12, *E. coli* XL1-Blue and probiotic *E. coli* Nissle 1917 across M-cell monolayers does not appear to be significantly different to translocation across Caco2-cl1 monolayers.

8. All Crohn's disease AIEC investigated translocated through M-cell monolayers to a greater extent than through Caco2-cl1 monolayers. Two of the five control subject AIEC *E. coli* translocated through M-cell monolayers to a greater extent than through Caco2-cl1 monolayers.

9. The translocation across M-cell monolayers of control *E. coli* K12 and Crohn's disease AIECs LF82, HM580, HM605 and HM615 was significantly inhibited by the presence of plantain NSP at 5 mg / mL. Plantain NSP also inhibited translocation of *Shigella sonnei* at 5 mg / mL, and *Salmonella* Typhimurium LT2 at 50 mg / mL across M-cell monolayers. Translocation of all investigated *E. coli, Shigella sonnei* and *Salmonella* Typhimurium LT2 through control Caco2-cl1 monolayers was not affected by the presence of plantain NSP, with the notable exception of *E. coli* HM615 which was found to be inhibited at the investigated concentration of 5 mg / mL. Plantain did not affect monolayer TEER.

10. Translocation of HM605 through both Caco2-cl1 and M-cell monolayers was significantly inhibited by broccoli NSP at 0.5, 5 and 50 mg / mL. Translocation of HM605 across Caco2-cl1 and M-cell monolayers was not affected by the presence of either leek or apple NSP at the investigated concentrations. The presence of broccoli, leek

or apple NSP did not significantly affect the TEER of either Caco2-cl1 or M-cell monolayers.

11. Polysorbate-80 at 0.01% v/v and polysorbate-60 at 0.1% v/v led to increased translocation of HM605 across Caco2-cl1 monolayers; polysorbate-80 at the tenfold higher concentration 0.1% v/v led to increased translocation across M-cell monolayers, whilst polysorbate-60 treatment had no effect upon translocation at the investigated concentrations. Monolayer TEER was not significantly affected by the presence of polysorbates.

12. Colonic mucosa-associated control patient AIEC and Crohn's disease AIEC are better able to survive and replicate within J774-A1 macrophages than the laboratory strain *E. coli* K12, as is the ileal Crohn's disease AIEC LF82. AIEC HM605 survives and replicates within membrane bound structures which resemble phagolysosomes.

13. At C_{max}, combination antibiotic treatment using ciprofloxacin, trimethoprim and either tetracycline or rifampin confers no increase in bacterial killing compared with ciprofloxacin treatment alone. At 10% C_{max}, using ciprofloxacin with tetracycline leads to increased bacterial killing compared to ciprofloxacin treatment alone. The most effective intramacrophage killing of HM605 at 10% C_{max} was seen using the antibiotic combination of ciprofloxacin with tetracycline and clarithromycin.

Chapter 8

Discussion

8.1 CONCLUDING DISCUSSION

The pathogenesis of Crohn's disease is incompletely understood. The increased presence of mucosa-associated Adherent and Invasive *E. coli* (AIEC) in Crohn's disease has now been well documented [Darfeuille-Michaud *et al.* 2004; Martin *et al.* 2004; Ryan *et al.* 2004; Mylonaki *et al.* 2005; Swidsinski *et al.* 2005; 2006; Baumgart *et al.* 2007; Kotlowski *et al.* 2007; Sasaki *et al.* 2007]. A causative role for these AIEC has not been established; certainly *in vitro,* they have been shown to adhere and invade epithelial cells, such as in Caco2-cl1 cells used here (Chapter 3), but how this correlates to the *in vivo* situation is unclear.

These AIEC are also now shown to translocate across *in vitro* derived M-cells, as a result of the work contained in this thesis. Whilst translocation of AIEC at M-cells correlates well with the location of the initial aphthoid ulcers observed in Crohn's disease patients, *implying* they are important in disease initiation, it is not evidence that AIEC *cause* the aphthoid ulcers characteristic of the disease.

E. coli have been observed *in vivo* in macrophages [Liu *et al.* 1995; Ryan *et al.* 2004]. *In vitro*, the replication of the typical ileal AIEC LF82 and several Liverpool colonic mucosa-associated AIEC isolates have already been observed with macrophages [Glasser *et al.* 2001; Subramanian *et al.* 2008].

Under normal circumstances, microbiota passing through the intestinal barrier would be expected to be cleared by a robust phagocytic system. In Crohn's disease however, defects in several cell types involved in the innate immune system have been reported to be associated with the disease. Cell types implicated include goblet cells [Podolsky *et al.* 1983; Van der Sluis *et al.*

2006], neutrophils [Nikolaus *et al.* 1998], eosinophils [Furuta *et al.* 2005], mast cells [He *et al.* 2004], Paneth cells [Wehkamp *et al.* 2004], macrophages [Marks *et al.* 2006] and dendritic cells

Paneth cells express NOD2 which is involved in the recognition of the bacterial peptidoglycan component muramyl dipeptide [Hugot *et al.* 2001; Lara *et al.* 2003; Ogura *et al.* 2001]; mutations in NOD2 have been implicated in Crohn's disease. Upon bacterial stimulation, Paneth cells produce antimicrobial molecules such as α-defensins [Ayabe *et al.* 2000]. Kobayashi *et al.* demonstrated that NOD2-deficient mice showed impaired Paneth cell responses upon challenge with muramyl dipeptide, and a failure to eradicate certain pathogens [Kobayashi *et al.* 2005]. Additionally, Crohn's disease patients have a diminished expression of α-defensins which is most pronounced in those with a NOD2 mutation [Wehkamp *et al.* 2004; Wehkamp *et al.* 2005]. A reduction in functional antimicrobial activity has also been reported for colonic biopsies isolated from Crohn's disease patients [Nuding *et al.* 2007].

In Crohn's disease, a reduction in function of macrophages has been reported, as has evidence of impaired neutrophil recruitment [Marks *et al.* 2006]. Monocyte-derived macrophages from Crohn's disease patients with a NOD2 mutation have been found to produce less of the chemoattractant IL-8 following stimulation with muramyl dipeptide, which in turn may lead to delayed recruitment of neutrophils and to suboptimal clearance of bacteria from the intestine, which has been reported by Marks *et al.*

Taken together, these findings lead to the hypothesis that, *in vivo*, AIEC are inadequately cleared by translocated across the epithelial barrier via the M-cells, are inadequately cleared by neutrophils, and become ingested by

macrophages where they replicate within endophagolysosomes and stimulate granuloma formation.

8.2 IMPLICATIONS FOR FUTURE STUDIES

Whilst the data presented here clearly show that Crohn's disease *E. coli* isolates are able to invade Caco2-cl1 cells, and work by others has shown these *E. coli* to lead to IL-8 release from epithelial cells *in vivo* [Subramanian et al. 2008]. The mechanism by which these *E. coli* invade epithelial cells is currently unknown. For LF82, type 1 pili-mediated adherence has been implicated in invasion [Boudeau *et al.* 2001], as has expression of bacterial flagella [Carvalho *et al.* 2008]. Deletion of the *yfgL* gene in strain LF82 resulted in a decreased ability to invade intestinal epithelial cells and a decreased release of outer membrane vesicles [Rolhion *et al.* 2005], as does deletion of the *NlpI* gene, which encodes a lipoprotein [Barnich *et al.* 2004]. The *yfgL* gene is involved in the synthesis and / or degradation process of peptidoglycan and in the susceptibility of *E. coli* strain K-12 to killing by glycolipid derivatives of vancomycin [Eggert *et al.* 2001]. To date, no biological role has been described for the NlpI lipoprotein.

Entry into intestinal mucosa by enteric pathogens *Salmonella* spp. and *Shigella* spp. is through the activation of host small Rho family GTPases Rac, Cdc42 and Rho. Rho-GTPase activation, via downsteam signalling events, results in a variety of host cell responses notably the formation of filopodia, lamellipodia and stress fibres. It is the regulation and reorganisation of the actin cytoskeleton in this way which results in phagocytosis [Jaffe *et al.* 2002]. It is possible that Crohn's disease AIEC also invade host-epithelia by a similar mechanism – my own preliminary data (submitted for the degree of M.Res,

2005) indicates that colonic mucosa-associated Crohn's disease *E. coli* isolate HM605 activates Rac-GTPase and Cdc42-GTPase in I407 cells, with a peak activation observed at 4 hours. Crohn's disease *E. coli* isolate HM427 activates Ras-GTPase in HT-29 cells within 5 minutes and sustains this activation for up to 60 minutes. This preliminary data suggests that therapeutic intervention using modulators of small GTPase activity may be beneficial to prevent bacterial uptake in the gut.

Understanding the mechanism by which AIEC invade epithelial cells *in vitro* may provide insight into their role in Crohn's disease *in vivo*.

The inhibition of translocation of AIEC across M-cells by plantain NSP is interesting, but raises the question of what exactly it is about the plantain NSP which inhibits the translocation of AIEC. It is probable that specific oligosaccharide structures within the plantain NSP interfere with bacteria – host cell interactions, and identifying these structures could lead to increased specificity to prevent bacterial translocation at M-cells. Mannose, a component sugar of plantain NSP (18.7% by wt), has already been shown to prevent LF82 adhesion to isolated Crohn's disease enterocytes but not control patient enterocytes when used at 2% (w / v) [Barnich *et al.* 2007].

That data presented here also have implications for diseases other than Crohn's disease. Plantain NSP has been shown to block adherence of Crohn's disease *E. coli* isolates to Caco2-cl1 cells *in vitro,* and to inhibit the translocation of Crohn's disease *E. coli* isolates across M-cell monolayers *in vitro.* Translocation of *Salmonella* Typhimurium LT2 and *Shigella sonnei* across M-cell monolayers is also blocked by plantain NSP in a dose-dependent fashion. Whilst work to date has focussed on Gram-negative organisms, the broad range of bacteria whose epithelial adhesion and M-cell translocation is

inhibited by soluble plantain NSP suggest the possibility that it may also be effective at preventing the adhesion to colonic epithelial cells or translocation across M-cells by Gram-positive bacteria.

One organism of particular interest is *Clostridium difficile*, a major cause of antibiotic-associated diarrhoea and colitis in humans. *C. difficile* is often considered to be a 'superbug' as it is notoriously difficult to eliminate. The cause of *C. difficile*-induced infection is considered to local release of enterotoxin (*toxin A*) and cytotoxin (*toxin B*) by *C. difficile* [Borriello et al. 1988; Lyerly et al. 1988; Riegler et al. 1995; Pothoulakis 1996; Taha et al. 2007]. Close proximity of *C. difficile* to the host epithelium is almost certainly necessary to produce toxic effects [Borriello *et al.* 1988], and preventing these interactions should therefore be of therapeutic benefit.

The prevention of adhesion of pathogenic bacteria to the intestinal epithelium by ingested soluble plant fibres seems likely to represent an important protective mechanism that has previously been overlooked.

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Appendices

APPENDIX 1 – BACTERIAL CHARACTERISTICS OF STRAINS SELECTED FOR STUDY WITHIN THIS THESIS

Bacterial Strain	Disease Group	Patient Number	Isolated from
E. coli HM95	Crohn's disease	15	Epithelial cell-associated
E. coli HM154	Crohn's disease	30	Intracellular
E. coli HM413	Crohn's disease	59	Mucus-associated
E. coli HM419	Crohn's disease	59	Epithelial cell-associated
E. coli HM580	Crohn's disease	71	Mucus-associated
E. coli HM605	Crohn's disease	73	Mucus-associated
E. coli HM615	Crohn's disease	74	Mucus-associated
E. coli LF82	Crohn's disease	----	----
E. coli HM428	Control (IBS/Polyposis)	60	Epithelial cell-associated
E. coli HM454	Control (IBS/Polyposis)	60	Mucus-associated
E. coli HM456	Control (IBS/Polyposis)	60	Mucus-associated
E. coli HM463	Control (IBS/Polyposis)	62	Mucus-associated
E. coli HM484	Control (IBS/Polyposis)	65	Epithelial cell-associated
E. coli HM488	Control (IBS/Polyposis)	65	Mucus-associated
E. coli K12	----	----	----
E. coli Nissle 1917	----	----	----
Salmonella Typhimurium LT2	----	----	----
Shigella sonnei	----	----	----

Bacterial Strain	API 20E [1]	Haemagglutination status [2]	Induces IL-8 [3]
E. coli HM95	5144552	-	+
E. coli HM154	5104572	-	+
E. coli HM413	7144552	-	n/a
E. coli HM419	5044552	+	n/a
E. coli HM580	4044512	+	+
E. coli HM605	5144552	+	+
E. coli HM615	5144152	+	+
E. coli LF82	n/a	-	n/a
E. coli HM428	5144512	+	+
E. coli HM454	5144512	+	n/a
E. coli HM456	5144512	+	n/a
E. coli HM463	5144572	-	n/a
E. coli HM484	7144552	-	+
E. coli HM488	5144552	-	n/a
E. coli K12	n/a	n/a	n/a
E. coli Nissle 1917	n/a	n/a	n/a
Salmonella Typhimurium LT2	n/a	n/a	n/a
Shigella sonnei	n/a	n/a	n/a

n/a – data not available

[1] Gram-negavtive bacteria identified by API 20E bacterial identification system (BioMerieux, Marcy L'etole, France) [Martin 2004]

[2] Determined by the ability to haemagglutinate red blood cells (taken from [Subramanian *et al.* 2008]

[3] Significant increase in IL-8 release from HT-29 colon epithelial cells compared to basal IL-8 release [Subramanian *et al.* 2008].

Bacterial Strain	Afa/Dr cluster [4]	Curli fimbriae [5]	Adherence to I407 cells	Invasion of I407 cells
E. coli HM95	-	+	+	+
E. coli HM154	-	+	+	+
E. coli HM413	n/a	n/a	+	+
E. coli HM419	n/a	n/a	+	+
E. coli HM580	+	-	+	+
E. coli HM605	-	+	+	+
E. coli HM615	-	+	+	+
E. coli LF82	n/a	n/a	+	+
E. coli HM428	n/a	n/a	+	+
E. coli HM454	-	+	+	+
E. coli HM456	-	+	+	+
E. coli HM463			+	+
E. coli HM484	n/a	n/a	+	+
E. coli HM488	-	-	+	+
E. coli K12	n/a	n/a	+	+
E. coli Nissle 1917	n/a	n/a	+	+
Salmonella Typhimurium LT2	n/a	n/a	+	+
Shigella sonnei	n/a	n/a	+	+

[4] Data from [Martin *et al.* 2004]

[5] Data from [Subramanian *et al.* 2008]

Bacterial Strain	Adherence to HT-29 cells	Invasion of HT-29 cells
E. coli HM95	+	+
E. coli HM154	+	-
E. coli HM413	+	+
E. coli HM419	+	+
E. coli HM580	+	+
E. coli HM605	+	-
E. coli HM615	+	-
E. coli LF82	n/a	n/a
E. coli HM428	n/a	n/a
E. coli HM454	n/a	n/a
E. coli HM456	n/a	n/a
E. coli HM463	+	-
E. coli HM484	+	+
E. coli HM488	n/a	n/a
E. coli K12	n/a	n/a
E. coli Nissle 1917	n/a	n/a
Salmonella Typhimurium LT2	n/a	n/a
Shigella sonnei	n/a	n/a

Bacterial Strain	Invasion into Caco2 cells [6]	Invasion into Caco2-cl cells [7]
E. coli HM95	< 1	2.72 ± 0.52
E. coli HM154	< 1	4.27 ± 1.22
E. coli HM413	n/a	n/a
E. coli HM419	4.39 ± 1.37	4.19 ± 1.74
E. coli HM580	< 1	3.64 ± 0.29
E. coli HM605	15.92 ± 2.74	10.04 ± 0.65
E. coli HM615	2.16 ± 0.11	3.32 ± 1.21
E. coli LF82	2.01 ± 0.83	1.58 ± 0.06
E. coli HM428	8.85 ± 2.82	12.29 ± 1.99
E. coli HM454	17.29 ± 3.18	11.71 ± 1.57
E. coli HM456	5.89 ± 0.92	8.45 ± 0.20
E. coli HM463	22.53 ± 3.92	25.55 ± 1.74
E. coli HM484	< 1	5.86 ± 0.57
E. coli HM488	2.79 ± 1.09	4.85 ± 0.34
E. coli K12	1	1
E. coli Nissle 1917	n/a	n/a
Salmonella Typhimurium LT2	> 1	> 1
Shigella sonnei	> 1	> 1

[6] Data expressed as invasion relative to *E. coli* K12

[7] Data expressed as invasion relative to *E. coli* K12

Bacterial Strain	Invasion of Caco2-cl cells inhibited by 50 mg / mL plantain NSP	Adhesion to Caco2-cl cells inhibited by 5 or 50 mg / mL plantain NSP
E. coli HM95	n/a	n/a
E. coli HM154	n/a	n/a
E. coli HM413	n/a	n/a
E. coli HM419	n/a	n/a
E. coli HM580	+	+
E. coli HM605	+	+
E. coli HM615	+	+
E. coli LF82	+	+
E. coli HM428	n/a	n/a
E. coli HM454	n/a	n/a
E. coli HM456	n/a	n/a
E. coli HM463	n/a	n/a
E. coli HM484	n/a	n/a
E. coli HM488	n/a	n/a
E. coli K12	+	+
E. coli Nissle 1917	n/a	n/a
Salmonella Typhimurium LT2	+	+
Shigella sonnei	+	+

Bacterial Strain	Phylotype [8]	Replication within macrophages
E. coli HM95	A	+
E. coli HM154	B1	+
E. coli HM413	n/a	+
E. coli HM419	n/a	+
E. coli HM580	D	+
E. coli HM605	B2	+
E. coli HM615	B2	+
E. coli LF82	B2	+
E. coli HM428	n/a	+
E. coli HM454	B2	+
E. coli HM456	B2	+
E. coli HM463	n/a	+
E. coli HM484	n/a	+
E. coli HM488	B2	+
E. coli K12	n/a	+
E. coli Nissle 1917	n/a	n/a
Salmonella Typhimurium LT2	n/a	n/a
Shigella sonnei	n/a	n/a

[8] Phylotype data from [Subramanian *et al.* 2008], analysis based on the findings of [Kotlowski *et al.* 2007].

APPENDIX 2 – SOLUTIONS

10% Resolving Polyacrylamide Gel (Immunoblotting)

21.6 mL	Distilled water
11.25 mL	Acrylamide/bis-acrylamide, 40% solution (SIGMA)
11.25 mL	1.5 M Tris buffer (pH 8.8)
450 µL	10% (w/v) SDS
450 µL	10% (w/v) Ammonium persulfate
45 µL	TEMED

4% Stacking Polyacrylamide Gel (Immunoblotting)

18.9 mL	Distilled Water
3 mL	Acrylamide/bis-acrylamide, 40% solution (Sigma Ltd)
7.5 mL	1.5 M Tris buffer (pH 8.8)
300 µL	10% (w/v) SDS
300 µL	10% (w/v) Ammonium persulfate
30 µL	TEMED

Luria Burtani agar

5g	Yeast extract
10g	Tryptone
10g	NaCl
15g	Agar
1L	Distilled water

Luria Burtani culture broth

5g	Yeast extract
10g	Tryptone
10g	NaCl
1L	Distilled Water

Phosphate Buffered Saline (PBS)

8 g	NaCl
0.2 g	KCl
1.44 g	Na_2HPO_4
0.24 g	KH_2PO_4
1 L	Distilled Water

Adjust pH to 7.4

Reynards Lead Citrate

1.33 g	Lead nitrate
1.76 g	Sodium citrate
8 mL	1M NaOH
42 mL	Distilled water

Uranyl Aceteate

1.25g	Uranyl acetate
12.5 mL	Ethanol
12.5 mL	Distilled water

SDS-PAGE running buffer

1 L	Distilled water
14.4 g	Glycine
3.03 g	Tris base
1 g	Sodium dodecyl sulphate

SDS-PAGE transfer buffer

800 mL	Distilled water
14.4 g	Glycine
3.03 g	Tris base
200 mL	Methanol

5x SDS-PAGE sample buffer

4 mL	Distilled water
1 mL	0.5 M Tris-HCl
0.8 mL	Glycerol
1.6 mL	10% SDS
0.4 mL	Beta-mercaptoethanol
0.2 mL	0.05% (w/v) bromophenol blue